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NOTE

CONTINUOUS HYDROGEN PEROXIDE PRODUCTION BY ORGANIC BUFFERS IN PHYTOPLANKTON CULTURE MEDIA[1]

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We investigated the production of hydrogen peroxide (HOOH) in illuminated seawater media containing a variety of zwitterionic buffers. Production rates varied extensively among buffers, with 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid (HEPES) highest and N-Tris(hydroxymethyl) methyl-3-aminopropanesulfonic acid (TAPS) among the lowest. The rate of HOOH accumulation was remarkably consistent over many days, and increased linearly with buffer concentration, natural seawater concentration, and light level. Concentrations of HEPES commonly used in culture media (1–10 mM) generated enough HOOH to kill the axenic *Prochlorococcus* strain VOL1 during growth in enriched seawater media at lower, environmentally realistic cell concentrations and/or under high light exposure. We also demonstrated that HEPES can be used experimentally to study the biological effects of chronic exposure to sublethal levels of HOOH such as may be experienced by light-exposed microorganisms.

Key index words: **buffer; culture media; HEPES; hydrogen peroxide; oxidative stress; pH; *Prochlorococcus*; ROS generation**

***Abbreviations*: DOC, dissolved organic carbon; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HOOH, hydrogen peroxide; MOPS, 3-(N-morpholino) propanesulfonic acid; ROS, reactive oxygen species; TAPS, N-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid**

HOOH is formed continuously in sunlit natural waters by the photooxidation of DOC compounds (Cooper et al. 1988). HOOH and related reactive oxygen species (ROS) are toxic, and therefore aerobic organisms deploy a number of chemical and enzymatic defenses to protect themselves (Imlay 2003). Because the submicromolar concentrations of HOOH found in most surface waters (e.g., Zika et al. 1985, Cooper and Lean 1989, Yuan and Shiller 2001, 2005, Avery et al. 2005) are well below harmful levels for commonly studied microorganisms such as *Escherichia coli* (Yoshpepurer and Henis 1976) and *Synechococcus elongatus* (Perelman et al. 2003), research on environmental HOOH has mainly focused on its roles in geochemical cycling, particularly in controlling the speciation of trace metals and the lability of dissolved organic carbon (DOC) (Mopper et al. 1991, Willey et al. 1999, Scully et al. 2003). However, a number of studies suggest that microbes (including important phytoplankters such as *Prochlorococcus*) in their natural habitats might be sensitive to HOOH concentrations within the natural range (Xenopoulos and Bird 1997, Drabkova et al. 2007, Morris et al. 2011).

Unfortunately, studying the effects of HOOH on cultured microbes is complicated by the presence of HOOH in culture media, arising from a number of sources, including autoclaving (Bogosian et al. 2000) and light exposure (Grzelak et al. 2001). One often overlooked source of HOOH in phytoplankton culture media is the buffer. Zwitterionic buffers such as HEPES, 3-(N-morpholino) propanesulfonic acid (MOPS), and TAPS are often considered optimal choices for such media (McFadden and Melkonian 1986, Harrison and Berges 2005, Watanabe 2005): they are effective within the pH range of natural waters (e.g., 7.0–8.5), and early experiments characterized them as chemically unreactive under physiological conditions, unusable as carbon sources for heterotrophic bacteria, and transparent in the UV-VIS range (Good et al. 1966). Contrary to these initial observations, subsequent studies have shown that one of the buffers described by Good et al. (1966), HEPES, is not chemically inert, but rather forms HOOH in light-exposed media at rates high enough to kill eukaryotic cell cultures (Zigler et al. 1985, Lepe-Zuniga et al. 1987).

In light of our discovery of the HOOH-sensitivity of the cyanobacterium *Prochlorococcus* (Morris et al.

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2011), we sought to confirm the original findings of Zigler et al. (1985) in the context of algal growth media, as well as to explore alternatives to HEPES for buffering. During the course of these experiments, we also discovered that HEPES may be exploited to deliver a controlled flux of HOOH to illuminated culture media for studies of the effects of chronic sublethal exposure to HOOH.

Using a chemiluminescence method described previously (Morris et al. 2011), we tested a number of buffers with pKa values in the relevant range for seawater media (Table S1 in the Supporting Information) for the ability to produce HOOH in natural or artificial seawater when exposed to light. Control tubes lacking light, buffer, or the natural/artificial seawater solutes (i.e., buffer prepared in milli-Q water) did not generate HOOH (slope of regression of HOOH on time not significantly greater than 0 based on a one-tailed, one-sample *t*-test, Fig. S1 in the Supporting Information). In contrast, most of the other buffers produced HOOH when exposed to light in a seawater matrix (Figs. 1A and Fig. S2 in the Supporting Information). Following a period of rapid HOOH formation over the first few hours, the rate decreased to a level that was sustained for many days (Fig. 1B). The zero-order rate constant of HOOH accumulation during this latter period (k_{HOOH}) varied dramatically based on the buffer used and whether natural or artificial seawater was used as a basal medium (Fig. 1C). In natural seawater, HEPES and DIPSO were comparable, and produced almost twice as much HOOH per hour as MOPS, four times as much as tricine, and almost an order of magnitude more than bicine or TAPS. The general pattern was similar in artificial seawater, but with much lower k_{HOOH} values in each case. On the other hand, some buffers did not detectably generate HOOH. The regression slope of HOOH on time was not significantly greater than 0 (one-tailed, one-sample *t*-tests) in light-exposed TAPS-buffered artificial seawater (Fig. 1C) or in Tris- and glycylglycine-buffered natural seawater (Fig. S2). Indeed, glycylglycine appeared to actively remove HOOH from the medium.

Depletion of O_2 in the medium using a catalyst and a sealed anaerobic chamber (see Appendix S1 in the Supporting Information) drastically lowered k_{HOOH} for HEPES and TAPS (Fig. S3 in the Supporting Information), confirming that O_2 is necessary for HOOH production in these systems. To establish the relative contribution of buffer, seawater solutes, and light to k_{HOOH}, we manipulated each separately from a "reference condition" of 1 mM HEPES, 65 μmol photons $\cdot m^{-2} \cdot s^{-1}$, and 100% natural seawater and measured k_{HOOH} over 3–7 d (Fig. 2). The regression of k_{HOOH} on seawater concentration and light flux was linear, with the latter exerting a greater effect on k_{HOOH} within the range of our experiments. HEPES concentrations from 0.01 to 1 mM were also strongly correlated with k_{HOOH} following log transformation of the data (Fig. 2A).

For several buffers, HOOH production eventually slowed and after several weeks HOOH concentrations appeared to approach an asymptote (e.g., the 10 mM HEPES curve in Fig. 1A and several curves in Fig. S2, indicated by black arrows). In other cases, HOOH concentrations began to decrease during extended incubations. This phenomenon was not universally observed even between replicates, suggesting it might be caused by a non-biological, HOOH-consuming contaminant such as Fe, possibly introduced during the sampling process.

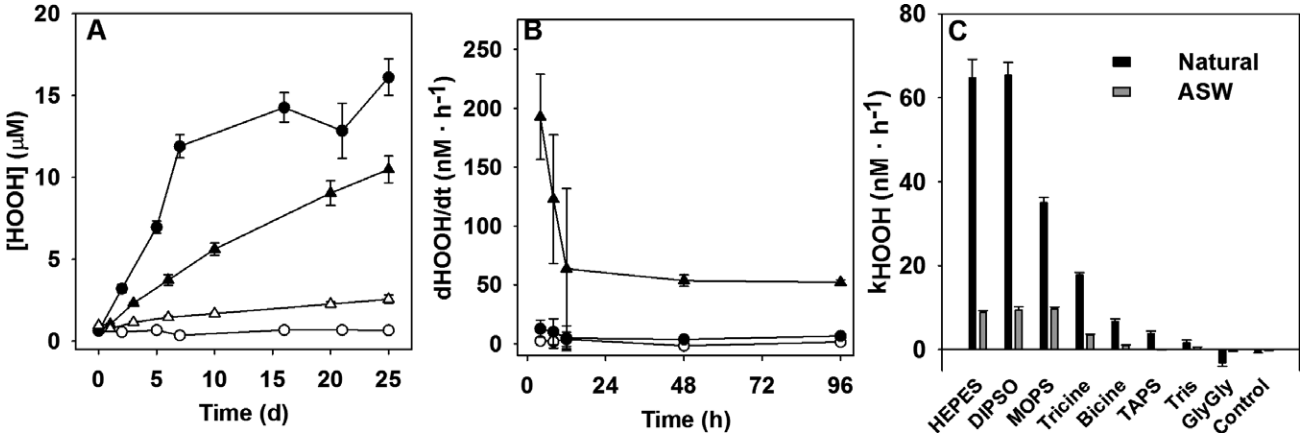

FIG. 1. Kinetics of HOOH formation in illuminated buffered seawater media. (A) [HOOH] over time in media buffered with 10 (closed circles), 1 (closed triangles), 0.1 (open triangles), or 0 mM (open circles) HEPES and exposed to light (65 μmol photons $\cdot m^{-2} \cdot s^{-1}$). (B) First derivative of HOOH concentration with respect to time for media containing either 10 mM HEPES (closed triangles) or TAPS (closed circles), or no buffer (open circles). Time zero indicates initial exposure to light at 65 μmol photons $\cdot m^{-2} \cdot s^{-1}$. (C) The long-term zero-order rate constants for HOOH formation (k_{HOOH}) in sterile, Chelexed natural seawater (black bars) or Turk's Island artificial seawater mix (gray bars) under 40 μmol photons $\cdot m^{-2} \cdot s^{-1}$ for a variety of buffers at 10 mM concentration. GlyGly, glycylglycine. Error bars in each panel are the SE of three replicates.

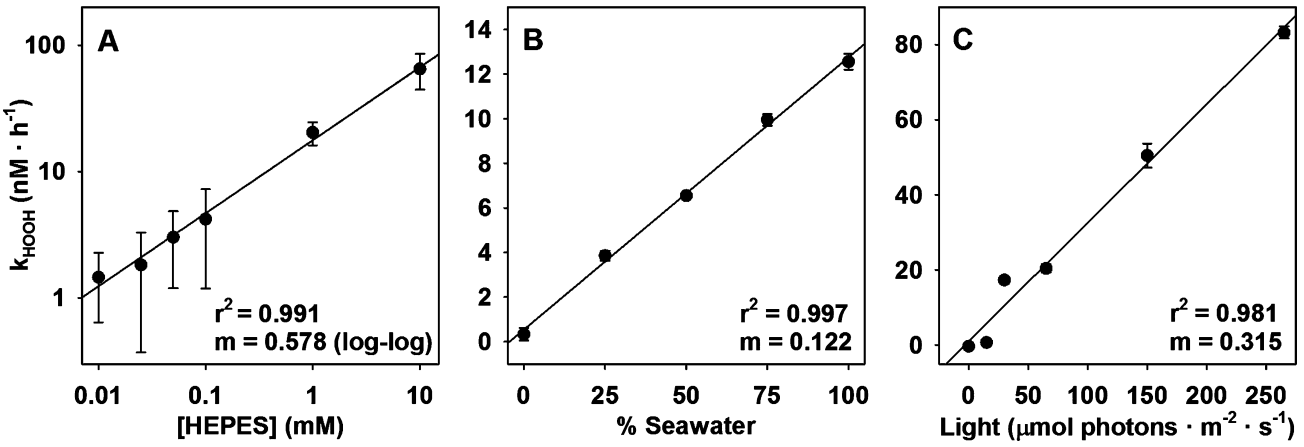

FIG. 2. Effects on k_{HOOH} by altering one of three parameters (A, buffer concentration; B, % natural seawater; C, light intensity) from the standard conditions of 1 mM HEPES, 65 μmol photons · m^{-2} · s^{-1}, and 100% natural seawater. Error bars are the SE of three averaged replicates. Black lines are linear regressions of the data. The line in A is a regression of the data following log-log transformation. m, slope of the regression of k_{HOOH} on the *x*-axis value.

During these studies, we noted that common microbiological methods could also generate HOOH in buffer-treated media. Buffered media in bottles stored under ambient lab lighting (~30 μmol photons · m^{-2} · s^{-1}, 12 h a day) for 1 week were universally lethal to *Prochlorococcus* cultures (inocula = 10^6 cells · mL^{-1}) regardless of incubation conditions. This effect was observed for both HEPES and TAPS-buffered media, but was eliminated completely by the addition of catalase prior to inoculation (data not shown). Using a bunsen burner to flame culture tubes during transfer procedures also generated HOOH. While quick exposure to the flame had minimal impact, excessive flaming generated as much as 2 μM HOOH (data not shown).

The unicellular cyanobacterium *Prochlorococcus* has been shown to have a density-dependent vulnerability to HOOH (Morris et al. 2011), and so the impact of buffer-generated HOOH was tested at several cell densities. Under continuous light at a moderate level of 65 μmol photons · m^{-2} · s^{-1}– equivalent to ~3% of the irradiance at the ocean surface – 1 mM HEPES was sufficient to prevent growth of *Prochlorococcus* strain VOL1 even at a relatively high inoculum of 10^6 cells · mL^{-1} (Fig. 3A, first set of bars). Conversely, TAPS-treated cultures were not significantly different from untreated controls, consistent with the lower k_{HOOH} of TAPS relative to HEPES. Addition of 1 unit · mL^{-1} catalase to HEPES cultures completely restored growth, confirming that there is no HEPES-based toxicity independent of HOOH formation.

Under a more natural light:dark cycle, HEPES and TAPS had variable effects on VOL1 growth at the high (10^6 cells · mL^{-1}) inoculum depending on buffer concentration, light level, and the presence of catalase. 1 mM HEPES or TAPS did not affect growth at moderate or high light levels (65 or 250 μmol photons · m^{-2} · s^{-1}, respectively; Fig. 3A). When the buffer concentration was raised to 10 mM, however, pronounced growth rate decreases were observed for all cultures. Both TAPS and HEPES lowered growth rates under moderate light, suggesting that even the lower rate of HOOH production by TAPS became deleterious at this buffer concentration. At high light (250 μmol photons · m^{-2} · s^{-1}), no HEPES cultures survived, whereas most TAPS cultures did. Catalase exerted a clear protective effect for most of these 10 mM buffer treatments, again demonstrating that the effect of these buffers on VOL1 growth was due to HOOH production. Catalase-treated TAPS cultures had higher growth rates than untreated cultures at both moderate and high light. Catalase also prevented HEPES-dependent mortality at low light, but could not restore growth in the presence of 10 mM HEPES at high light.

When the initial inoculum of *Prochlorococcus* was lowered to the environmentally realistic level of 10^5 cells · mL^{-1} the effects of the buffers on growth became even more pronounced (Fig. 3B). Under these conditions no HEPES-containing cultures grew, regardless of light level, buffer concentration, or the presence of catalase. On the other hand, 1 mM TAPS cultures continued to grow normally at moderate light, although only one of three replicates grew at high light. The addition of catalase restored the control growth rate to the 1 mM TAPS cultures grown at high light. Strikingly, no cultures with an ecologically relevant inoculum survived in the presence of 10 mM buffer at either light level, even with catalase.

The HEPES toxicity described here for an axenic strain of *Prochlorococcus* is consistent with other studies demonstrating HEPES toxicity in algal cultures (Ferguson et al. 1980, Blanchemain et al. 1994) and suggests that the observed toxicity may be due to HOOH production in those illuminated cultures as

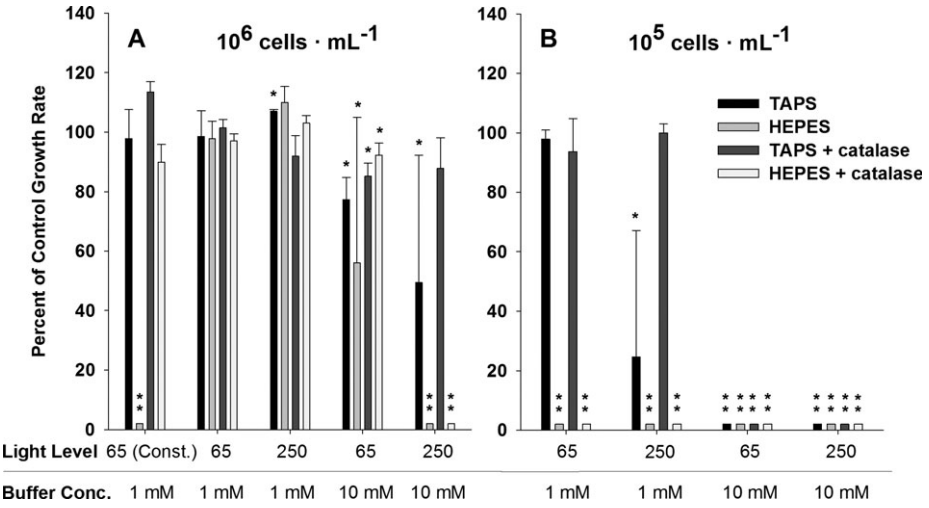

FIG. 3. Effects of buffers on the growth rate of *Prochlorococcus* VOL1. Cultures were grown under either moderate or high light intensities (indicated values are in μmol photons · m^{-2} · s^{-1}). Most cultures were grown under a 12:12 light:dark photoperiod; the first set of bars in graph A were grown under a constant light regime. Cultures were initially inoculated with 10^6 (A) or 10^5 (B) cells · mL^{-1} of VOL1. All growth rates are expressed as a percentage of the growth rate of a simultaneously inoculated control (without buffer) culture. Single asterisks indicate the samples are significantly different from the relevant control either based on unpaired *t*-tests ($P < 0.05$) or because one or more test cultures failed to grow. Double asterisks indicate absence of detectable growth after 60 d in all three replicate cultures. Error bars are the SD of three replicate cultures.

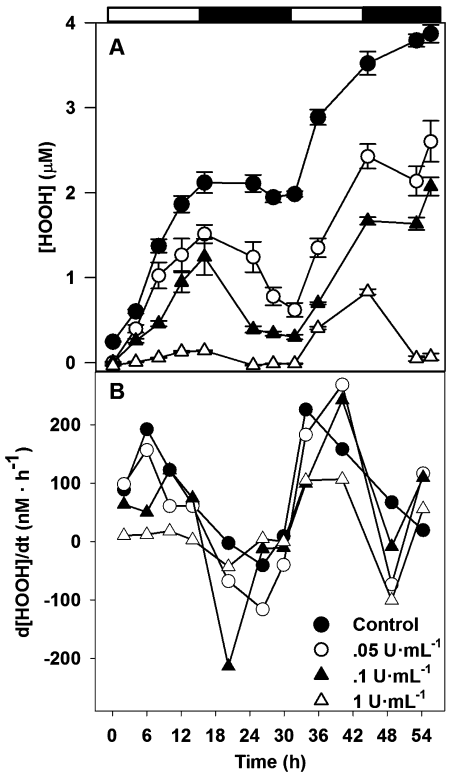

FIG. 4. (A) Time series of HOOH accumulation in natural seawater with 10 mM HEPES either with or without catalase (see legend in B). Samples were incubated under a light:dark regime (16:16 on day 1, 12:12 on day 2, indicated by light and dark bars at the top of the figure). Error bars are the SE of three replicate treatments. (B) Derivatives of the data in A with respect to time. Each data point is the average change in [HOOH] between the two flanking time points in A.

well. TAPS is the preferred buffer in dinoflagellate media (Loeblich 1975), and our results with TAPS are consistent with that report. While we readily acknowledge that both *Prochlorococcus* (Rippka et al. 2000, Moore et al. 2007) and diverse fresh- and saltwater eukaryotic microorganisms (McFadden and Melkonian 1986) have been successfully cultured in HEPES-containing media, we suspect that special handling of these cultures (e.g., high inoculum concentrations, lower light levels, presence of heterotrophic contaminants) or high intrinsic HOOH resistance of the organisms facilitated their survival.

Even in cases where organisms have apparently normal growth, however, it is important to keep in mind that the presence of Good's buffers in culture media produces a constant flux of ROS whenever the media are exposed to light. Although this flux may not be sufficient to result in cell death or growth arrest, it may still alter gene expression, metabolism, and cell composition (Hidalgo and Demple 1996, Godon et al. 1998, Dummermuth et al. 2003, Apel and Hirt 2004, Latifi et al. 2009), and may complicate interpretations of experimental results. As an example, *E. coli* exhibits an oxidative stress response when first exposed to commonly used rich media agar plates (Cuny et al. 2007), which are known to contain HOOH (Bogosian et al. 2000). Thus, for situations where it is necessary to add a buffer to a phytoplankton growth medium (McFadden and Melkonian 1986, Harrison and Berges 2005, Watanabe 2005) – for *Prochlorococcus*, this would include artificial seawater as well as high-volume natural seawater media (Rippka et al. 2000, Moore et al. 2007) – this study provides a number

of procedural improvements to avoid their deleterious side effects. We offer these suggestions in the Appendix S2 in the Supporting Information.

While in some situations in nature microorganisms are exposed to acute, lethal levels of ROS (e.g., rainfall events, oxidative bursts inside macrophages), a more common occurrence may be chronic exposure to lower, sublethal levels (e.g., in corals and their symbionts [Richier et al. 2005]). A technical challenge for the study of these chronic sublethal exposures is to provide a continuous supply of HOOH, as a single dosage applied at the initiation of the experiment may only reveal acute effects. A better strategy would be to provide a continuous flux of HOOH to a culture, perhaps employing a chemostat apparatus or certain enzymatic reactions (e.g., glucose oxidase [Antunes and Cadenas 2001]) to do so. However, these methods are technically challenging and, moreover, difficult to employ for slower growing organisms such as many phytoplankton. While the production of HOOH by HEPES in light-exposed media makes it problematic as a general-use buffer for phototrophic cultures, we reasoned that HEPES could provide a simple, adjustable means to provide a continuous flux to cultures for the study of long-term sublethal exposures to HOOH. The net accumulation of HOOH in HEPES-treated media is linear over at least several days and potentially much longer: even at the highest rate of production we measured, ~1.5 μM HOOH per day, it would take over 4 years to degrade 25% of the HEPES. Furthermore, the level of HOOH production may be fine-tuned by adjusting any of the three parameters measured here: seawater concentration, buffer concentration, or light level. By adjusting the ratio of TAPS:HEPES, it may be possible to separate the effect of increased HOOH production from any effect obtained from changing the buffering capacity of the culture media.

To demonstrate the utility of this system, we used HEPES in conjunction with catalase in a light/dark incubator to simulate the diel cycle in HOOH concentrations observed in some natural waters. In catalase-free buffered samples, HOOH accumulated at a consistent rate during two consecutive light periods (Fig. 4A) and followed the kinetics described above (Fig. 1B). HOOH accumulation ceased at "night" and resumed immediately upon illumination in the "morning," with an initially high rate that gradually diminished (Fig. 4B). In catalase-treated samples, net HOOH accumulation was attenuated as a function of catalase concentration, with net HOOH accumulation in the light and net HOOH removal over the dark period (Fig. 4B). However, the degree of attenuation and night-time consumption decreased on day 2 relative to day 1, suggesting a loss of catalase activity over the course of the experiment. This is consistent with previous reports indicating that catalase is itself photosensitive (Tytler et al. 1984, Morris et al. 2011). Nevertheless, it appears that 0.1–1 unit $\cdot$ mL^{-1} of catalase may be used in conjunction with HEPES to simulate the HOOH periodicity observed in natural waters, at least for the first 1 or 2 d.

In summary, we have demonstrated that several buffers can cause the rapid accumulation of HOOH in illuminated culture media. The impacts of such HOOH production could be extreme, as the case for *Prochlorococcus*, or more subtle, for organisms with a more robust ROS defense system (e.g., Hidalgo and Demple 1996, Godon et al. 1998, Dummermuth et al. 2003, Apel and Hirt 2004, Latifi et al. 2009). In either case, we feel that it is important to recognize that illuminated phytoplankton culture media that utilize a buffer to control pH may subject the cultures to a dynamic, increasing concentration of HOOH. Accordingly, we have replaced HEPES with TAPS in our artificial seawater medium for growing *Prochlorococcus*. Finally, we have demonstrated the potential for exploiting HEPES to provide controlled, gradual increases in HOOH, which can be used to assess the physiological response to chronic sublethal exposures of this oxidant.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. HOOH production in buffer-treated media incubated (A) in the dark or (B) in light-exposed milli-Q water (i.e., without seawater solutes). Buffers are at 10 mM. Error bars are the SE of three replicate treatments.

Figure S2. HOOH dynamics over time for illuminated natural seawater treated with a variety of buffers (at 10 mM). Plotted are the results of individual samples. Black arrows indicate "asymptotes," discussed in the text.

Figure S3. A comparison of the long-term HOOH production rate in buffered media with and without the presence of O_2. Error bars are the SE of three replicate samples.

Table S1. Buffers studied in this work.

Table S2. Multiple linear regression of k_{HOOH} versus medium characteristics.

Appendix S1. Detailed methods.

Appendix S2. Suggestions for preparation of phytoplankton growth media.