# Trace chemical species in marine incubation experiments, part A. Experiment design and bacterial abundance control extracellular H$_2$O$_2$ concentrations.

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## Abstract

The extracellular concentration of H$_2$O$_2$ in surface aquatic environments is controlled by a balance between photochemical production and the microbial synthesis of catalase and peroxidase enzymes to remove H$_2$O$_2$ from solution. In any kind of incubation experiment, the formation rates and equilibrium concentrations of ROS may be sensitive to both the experiment design (particularly to the regulation of incident light) and the abundance of different microbial groups (as both cellular H$_2$O$_2$ production and catalase/peroxidase enzyme production rates differ between species). Whilst there are extensive measurements of photochemical H$_2$O$_2$ formation rates and the distribution of H$_2$O$_2$ in the marine environment, it is poorly constrained how different microbial groups affect extracellular H$_2$O$_2$ concentrations, how comparable extracellular H$_2$O$_2$ concentrations within large scale incubation experiments are to those observed in the surface-mixed layer, and to what extent a miss-match with environmentally relevant concentrations of ROS in incubations could influence biological processes differently to what would be observed in nature. Here we show that both experiment design and bacterial abundance consistently exert control on extracellular H$_2$O$_2$ concentrations across a range of incubation experiments in diverse marine environments.

During 4 large scale (>1000 L) mesocosm experiments (in Gran Canaria, the Mediteranean, Patagonia and Svalbard) most experimental factors appeared to exert only minor, or no, direct effect on $H_2O_2$ concentrations. For example, in 3 of 4 experiments where pH was manipulated (to 0.4-0.5 below ambient pH) no significant change was evident in extracellular $H_2O_2$ concentrations relative to controls. An influence was sometimes inferred from zooplankton density, but not

5 consistently between different incubation experiments and no change in $H_2O_2$ was evident in controlled experiments using different densities of the copepod *Calanus finmarchichus* grazing on the diatom *Skeletonema costatum* (<1% change in $[H_2O_2]$ comparing copepod densities from 1-10 $L^{-1}$). Instead, the changes in $H_2O_2$ concentration contrasting high/low zooplankton incubations appeared to arise from the resulting changes in bacterial activity. The correlation between bacterial abundance and extracellular $H_2O_2$ was stronger in some incubations than others ($R^2$ range 0.09 to 0.55), yet high bacterial

10 densities were consistently associated with low $H_2O_2$. Nonetheless, the main control on $H_2O_2$ concentrations during incubation experiments relative to those in ambient, unenclosed waters was the regulation of incident light. In an open (lidless) mesocosm experiment in Gran Canaria, $H_2O_2$ was persistently elevated (2-6 fold) above ambient concentrations; whereas using closed high density polyethylene mesocosms in Crete, Svalbard and Patagonia $H_2O_2$ within incubations was always reduced (median 10-90%) relative to ambient waters.

15 **1.0 Introduction**

Reactive oxygen species (ROS), such as $H_2O_2$, are ubiquitous in surface aquatic environments due to photochemical formation (Van Baalen and Marler, 1966; Moore et al., 1993; Miller and Kester, 1994). $H_2O_2$ is present at concentrations on the order of 10-100 nM in the ocean's surface mixed layer with its concentration generally declining sharply with depth (Price et al., 1998; Yuan and Shiller, 2001; Gerringa et al., 2004). Because its decay rate is slow (observed half-lives in

20 seawater range from 10 to 120 h, Petasne and Zika 1997) compared to less stable ROS such as superoxide ($O_2^-$) and the hydroxyl radical ($OH^-$), extracellular $H_2O_2$ concentrations in surface waters show a pseudo-sinuous diurnal cycle, with elevated $H_2O_2$ concentrations occurring during daylight hours (Price et al., 1998).

$H_2O_2$ features as a reactive intermediate in the natural biogeochemical cycling of many compound groups including

25 halocarbons (Hughes and Sun, 2016), trace metals (Moffett and Zika, 1987; Voelker and Sulzberger, 1996; Hansel et al., 2015) and dissolved organic matter (DOM) (Cooper et al., 1988; Scully et al., 2003). Previous work has highlighted the susceptibility of a broad range of marine biota to elevated extracellular $H_2O_2$ concentrations (Bogosian et al., 2000; Morris et al., 2011) and argued that measurable negative effects on metabolism occur in some marine species at $H_2O_2$ concentrations within the range of ambient surface-mixed layer concentrations (Morris et al., 2011; Baltar et al., 2013). Peroxidase and

30 catalase enzymes are widely produced by marine microbes to lower extracellular $H_2O_2$ concentrations and these enzymes are the dominant sink for $H_2O_2$ in the surface marine environment (Moffett and Zafiriou, 1990; Angel et al., 1999). The reliance of some species including strains of *Prochlorococcus*, which do not produce such enzymes, on other 'helper' organisms to

remove extracellular $H_2O_2$ underpins a theory of reductive evolution, 'the Black Queen Hypothesis' (BQH) (Morris et al., 2012). BQH infers that because the removal of extracellular $H_2O_2$ by any species is a communal benefit, there is an energetic benefit to be gained to an individual species by losing genes associated with extracellular $H_2O_2$ detoxification. Loss of these genes continues to be favourable to individual species until only a minority of community members poses the ability to

5   remove $H_2O_2$, and the benefit of further loss would be offset by the negative effects of increasing extracellular $H_2O_2$ concentrations (Morris et al., 2012).

It is already acknowledged that laboratory incubation studies using buffered growth media are often conducted at $H_2O_2$ concentrations $2\text{-}10\times$ higher than those found in the surface ocean (Morris and Zinser, 2013). We have previously

10   hypothesized that the same may be generally true for meso-scale experiments (Hopwood et al., 2018) because the relative stability of $H_2O_2$ means that the enclosure of water at the ocean's surface within mesocosms can lead to elevated $H_2O_2$ concentrations. Yet there are presently few examples in the literature of incubation experiments where ROS concentrations are measured and therefore it is unknown how changes to other stressors, or changes to experimental design, affect extracellular ROS concentrations. In order to assess whether ROS could be a significant artefact in incubation experiments;

15   and to investigate how extracellular $H_2O_2$ concentrations respond to changes in DOC, pH and grazing pressure; here we collate data on $H_2O_2$ from a series of small to large scale (20-8000 L) incubation experiments with varying geographical location (Table 1).

## 2.0  Methods

### 2.1  Mesocosm set up and sampling

20   Eight incubation experiments (Table 1A) were constructed using coastal seawater which was either collected through pumping from small boats deployed offshore, or from the end of a floating jetty. Three of these incubations were outdoor mesocosm experiments (in Patagonia, Svalbard and the Mediterranean) conducted using the same basic setup (based on that used in earlier experiments described by Larsen et al., 2015). For these three mesocosms, 10 identical cubic high density polyethylene (HDPE) 1000-1500 L tanks were filled ~95% with seawater which was passed through nylon mesh (size as per

25   Table 1B) to remove mesozooplankton. The 10 closed mesocosm tanks were then held in position with a randomized treatment configuration and incubated at ambient seawater temperature. In Svalbard, Patagonia and Gran Canaria the mesocosms were tethered to a jetty. In the Mediterranean the mesocosms were held in a pool facility at the Hellenic Centre for Marine Research which was continuously flushed with seawater to maintain a constant temperature. An extra HDPE container (to which no additions were made) was also filled to provide an additional supply of un-manipulated seawater

30   (without zooplankton, DOC, or nutrient additions) for calibration purposes and baseline measurements on day 0. During the Mediterranean mesocosm, this surplus container was incubated alongside the mesocosms for the duration of the experiment without any further additions/manipulation.

| Label | Location | Month / year | Duration / days | Manipulated drivers | Scale / L | Site | Design Fig. S1 | $H_2O_2$ data available |
|---|---|---|---|---|---|---|---|---|
| MesoPat (Ocean Certain) Mesocosm | Comau fjord, Patagonia | Nov 2014 | 11 | DOC, grazing | 1000 | In-situ | I | Diurnal cycle. Limited time series |
| MesoPat (Ocean Certain) Multistressor | Comau fjord, Patagonia | Nov 2014 | 8 | DOC, grazing, pH | 20 | Temperature controlled room | II | Final $[H_2O_2]$ |
| MesoPat (Ocean Certain) Microcosm | Comau fjord, Patagonia | Nov 2014 | 11 | DOC, grazing | 20 | Temperature controlled room | III | Final $[H_2O_2]$ |
| MesoArc (Ocean Certain) Mesocosm | Kongsfjorden, Svalbard | July 2015 | 12 | DOC, grazing | 1250 | In-situ | I | Diurnal cycle |
| MesoArc (Ocean Certain) Multistressor | Kongsfjorden, Svalbard | July 2015 | 8 | DOC, grazing, pH | 20 | Temperature controlled room | II | Limited time series |
| MesoMed (Ocean Certain) Mesocosm | Hellenic Centre for Marine Research, Crete | May 2016 | 12 | DOC, grazing | 1500 | Outdoor temperature controlled pool | I | Diurnal cycle, $H_2O_2$ time series, decay rates, $H_2O_2$ spiked incubation |
| MesoMed (Ocean Certain) Multistressor | Hellenic Centre for Marine Research, Crete | May 2016 | 9 | DOC, grazing, pH | 20 | Temperature controlled room | II | Final $[H_2O_2]$ |
| Gran Canaria (The Future Ocean) Mesocosm | Taliarte Harbour, Gran Canaria | Mar 2016 | 28 | $pCO_2$ | 8000 | In-situ | IV | Diurnal cycle, $H_2O_2$ time series, $H_2O_2$ spiked incubation |

| Experiment | PAT (Patagonia) | ARC (Svalbard, Arctic) | MED (Crete, Mediteranean) | Gran Canaria |
|---|---|---|---|---|
| **Mesocosm** | | | | |
| Containers | HDPE 1000 L | HDPE 1250 L | HDPE 1500 L | Polyurethane 8000 L |
| Zooplankton treatment | +30 copepods $L^{-1}$ | +5 copepods $L^{-1}$ | +4 copepods $L^{-1}$ | NA |
| Macronutrient addition | N added as $NO_3$ | N added as $NH_4$ | N added as 50/50 $NH_4$/$NO_3$ | N added as $NO_3$ |
| Macronutrient addition timing | Daily | Daily | Daily | Day 18 only |
| Macronutrients added (per addition) | 1.0 µM $NO_3$, 1.0 µM Si, 0.07 µM $PO_4$ | 1.12 µM $NO_3$, 1.2 µM Si, 0.07 µM $PO_4$ (11.4 µM Si added on day 1) | 48 nM $NO_3$, 48 nM $NH_4$, 6 nM $PO_4$ | 3.1 µM $NO_3$, 1.5 µM Si, 0.2 µM $PO_4$ |
| Screening of initial seawater | NA | 200 µm | 140 µm | 3 mm |
| **Multistressor** | | | | |
| Containers | HDPE collapsible 20 L | HDPE collapsible 20 L | HDPE collapsible 20 L | |
| Zooplankton treatment | +30 copepods $L^{-1}$ | +5 copepods $L^{-1}$ | +4 copepods $L^{-1}$ | |
| Light regime | 15 h light / 9 h dark | 24 h light | 15 h light / 9 h dark | |
| Macronutrient addition | Same as Mesocosm | Same as Mesocosm | Same as Mesocosm | |
| Macronutrient addition timing | Daily | Daily | Daily | |
| Macronutrients added (per addition) | 1.0 µM $NO_3$, 1.0 µM Si, 0.07 µM $PO_4$ | 1.12 µM $NH_4$, 1.2 µM Si, 0.07 µM $PO_4$ | 48 nM $NO_3$, 48 nM $NH_4$, 6 nM $PO_4$ | |
| pH post adjustment | 7.54±0.09 | 7.76±0.03 | 7.64±0.02 | |
| pH pre-adjustment | 7.91±0.01 | 8.27±0.18 | 8.08±0.02 | |
| Screening of initial seawater | 200 µm | 200 µm | 140 µm | |
| Temperature / ℃ | 13-18 | 4.0-7.0 | 19.9-21.5 | |
| **Microcosm** | | | | |
| Containers | HDPE collapsible 20 L | | | |
| Grazing treatment | +30 copepods $L^{-1}$ | | | |
| Light regime | 15 h light / 9 h dark | | | |
| Macronutrient addition timing | Daily | | | |
| Macronutrient addition | Nitrogen was added as $NO_3$ | | | |
| Macronutrients added (per addition) | 1.0 µM $NO_3$, 1.0 µM Si, 0.07 µM $PO_4$ | | | |
| Screening of initial seawater | 200 µm | | | |
| Temperature / ℃ | 14-17 | | | |

**Table 1 (a) Details of experiments where $H_2O_2$ data were collected. Data from 8 separate experiments are presented, including 4 outdoor mesocosm experiments and 4 indoor microcosm/multistressor experiments. 'DOC' dissolved organic carbon. (b) Experiment details for each experiment. For a visual representation of experiment designs, the reader is referred to Supplementary Material. 'HDPE' high density polyethylene. 'NA' not applicable.**

5    The 10-mesocosm experiment design matrix was the same for all 3 Ocean Certain (MesoPat, MesoArc, MesoMed) mesocosms (Fig. S1, design I). For these 3 mesocosm experiments, zooplankton were collected one day in advance of requirement using horizontal tows at ~30 m depth with a mesh net equipped with a non-filtering cod end. Collected zooplankton were then stored overnight in 100 L containers and non-viable individuals removed by siphoning prior to making zooplankton additions to the mesocosm containers. After filling the mesocosms, zooplankton (quantities as per Table

10   1B) were then added to 5 of the containers to create contrasting high/low grazing conditions. Macronutrients ($NO_3$/$NH_4$, $PO_4$ and Si) were added to mesocosms daily (Table 1B). Across both the 5-high and 5-low grazing tank treatments, a dissolved organic carbon (DOC) gradient was created by addition of glucose to provide carbon at 0, 0.5, 1, 2 and 3 times the Redfield Ratio (Redfield, 1934) with respect to added $PO_4$. Mesocosm water was sampled through silicon tubing (permanently fixed into each mesocosm lid) immediately after mixing of the containers using plastic paddles (also mounted within the

15   mesocosms through the lids) with the first 2 L discarded in order to flush the sample tubing.

A 4th outdoor mesocosm experiment (Gran Canaria) used 8 cylindrical polyurethane bags with a depth of approximately 3 m, a starting volume of ~8000 L and no lid or screen on top (Hopwood et al., 2018). After filling with coastal seawater the bags were allowed to stand for 4 days. A pH gradient across the 8 tanks was then induced (on day 0) by the addition of varying

20   volumes of filtered, $pCO_2$ saturated seawater (resulting in $pCO_2$ concentrations from 400-1450 μatm, treatments outlined Fig. S1 IV) using a custom-made distribution device (Riebesell et al., 2013). A single macronutrient addition (3.1 μM nitrate, 1.5 μM silicic acid and 0.2 μM phosphate) was made on day 18 (Table 1B).

### 2.2 Microcosm and multistressor set up and sampling

A 10-treatment microcosm incubation mirroring the MesoPat 10 tank mesocosm (treatment design as per Fig. S1 I, but with

25   6 × 20 L containers per treatment -one for each time point- rather than a single HDPE tank) and three 16-treatment multistressor experiments (Fig. S1 II) were also conducted as part of the Ocean Certain project using artificial lighting in temperature controlled rooms (Table 1, Fig. S1). For all 3 multistressor incubations (Patagonia, Svalbard and Mediterranean) and the single microcosm incubation (Patagonia), coastal seawater (filtered through nylon mesh) was used to fill 20 L HDPE collapsible containers. The 20 L containers were arranged on custom made racks with light provided by a network of 36 W

30   lamps (Phillips, MASTER TL-D 90 De Luxe 36W/965 tubes). The number and orientation of lamps was adjusted to produce a light intensity of 80 μmol quanta $m^{-2}$ $s^{-1}$. A diurnal light regime representing spring/summer light conditions at each fieldsite was used and the tanks were agitated daily and after any additions (e.g. glucose, acid or macronutrient solutions) in order to ensure a homogeneous distribution of dissolved components. In all 20 L scale experiments, macronutrients were

added daily (as per Table 1B). One 20 L container from each treatment set was 'harvested' for sample water each sampling day.

The experiment matrix used for the microcosm incubation duplicated the MesoPat mesocosm design (Table 1B) and thereby consisted of 10 treatments. The experiment matrix for the 3 multistressor experiments (outlined in Fig. S1 II) duplicated the Ocean Certain mesocosm design (Fig. S1 I), with one less C/glucose treatment and an additional pH manipulation (Table 1B). The multistressor experiments thereby consisted of 16 treatments. pH manipulation was induced by adding a spike of HCl (trace metal grade) on day 0 only. Sample water from 20 L collapsible containers was extracted using a plastic syringe and silicon tubing which was mounted through the lid of each collapsible container.

Throughout, where changes in any incubation experiment are plotted against time, 'day 0' is defined as the day the experimental gradient (zooplankton, DOC, $pCO_2$) was imposed. Time prior to day 0 was intentionally introduced during some experiments to allow water to equilibrate with ambient physical conditions after container filling. $H_2O_2$ concentration varies on diurnal timescales and thus during each experiment where a time series of $H_2O_2$ concentration was measured, sample collection and analysis occurred at the same time daily ($\pm$ 0.5 h) and the order of sample collection was random.

### 2.3 Ancillary experiments

Four side experiments (1-4 below) were conducted to investigate potential links between bacterial/zooplankton abundance and extracellular $H_2O_2$ concentrations. Where specified, $H_2O_2$ concentrations were manipulated to form high, medium and low $H_2O_2$ conditions by adding aliquots of either a 1 mM $H_2O_2$ solution (prepared weekly from $H_2O_2$ stock) to increase $H_2O_2$ concentration, or bovine catalase (prepared immediately before use) to decrease $H_2O_2$ concentration. All treatments were triplicated. Catalase is photo-deactivated and biological activity to remove extracellular $H_2O_2$ follows the diurnal cycle (Angel et al., 1999; Morris et al., 2016), so catalase/$H_2O_2$ additions were conducted at sunset in order to minimize the additions required. Bovine catalase was used as received (Sigma Aldrich) with stock solutions prepared from frozen enzyme (stored at -20°C). De-natured catalase was prepared by heating enzyme solution to >90°C for 10 min.

(1) In Gran Canaria a 5 day experiment was conducted, using 5 L polypropylene bottles. After filling with offshore seawater, and the addition of macronutrients which matched the concentrations added to the Gran Canaria mesocosm (3.1 μM nitrate, 1.5 μM silicic acid and 0.2 μM phosphate), bottles were incubated under ambient light and temperature conditions within Taliarte Harbor. (2) In Crete, a similar 7 day incubation was conducted in the HCMR pool facility using 20 L HDPE containers. Seawater was extracted from the baseline MesoMed mesocosm (no DOC or zooplankton addition) on day 11 and then incubated without further additions except for $H_2O_2$ manipulation. After day 5 no further $H_2O_2$ manipulations were made. (3) As per (2), seawater was withdrawn from the baseline MesoMed mesocosm on day 11 and then incubated without further addition except for $H_2O_2$ manipulation in 500 mL trace metal clean LDPE bottles under the artificial lighting

conditions used for the MesoMed multistressor incubation. (4) A short term (20 h) experiment was conducted in trace metal clean 4 L HDPE collapsible containers to investigate the immediate effect of grazing on $H_2O_2$ concentrations. Filtered (0.2 µm, Satorius) coastal seawater (S 32.8, pH 7.9) water was stored in the dark for 3 days before use. The diatom *Skeletonema costatum* (NIVA-BAC 36 strain culture (CAA) from the Norsk Institutt for vannforskning (NIVA)) was used as a model

5    phytoplankton grown in standard f/2 medium (Guillard and Ryther, 1962). Each treatment consisted of a total volume of 2 L seawater and contained macronutrients, 7.5 ml of the original medium (resulting in an initial chlorophyll a concentration of 3 µg L$^{-1}$ in the incubations) and treated seawater containing the copepod *Calanus finmarchichus* corresponding to each desired density. The light regime was produced with fluorescent lighting with a mean luminous intensity of 80-90 µmol m$^{-2}$ s$^{-1}$ and the temperature maintained at 10.5-10.9°C.

Light levels during all Ocean Certain experiments (Table 1) were quantified using a planar Li-cor Q29891 sensor connected to a Li-cor Li-1400 data logger. Diurnal experiments measuring $H_2O_2$ concentrations in mesocosms or ambient surface (10 cm depth) seawater were conducted using flow injection apparatus with a continuous flow of seawater into the instrument through a PTFE line as described previously (Hopwood et al., 2018). For extensive datasets, the diurnal range of $H_2O_2$

15    concentrations was determined as the difference between the means of the highest and lowest 10% of datapoints.

### 2.4 Chemical analysis

**$H_2O_2$**

$H_2O_2$ samples were collected in opaque HDPE 125 mL bottles (Nalgene) which were pre-cleaned (1 day soak in detergent, 1 week soak in 1 M HCl, 3 rinses with de-ionized water) and dried under a laminar flow hood prior to use. Bottles were rinsed

20    once with sample water, filled with no headspace and always analysed within 2 h of collection via flow injection analysis (FIA) using the Co(II) catalysed oxidation of luminol (Yuan and Shiller, 1999). FIA systems were assembled and operated exactly as per Hopwood et al., (2017) producing a detection limit of < 1 nM. Calibrations were run daily and with every new reagent batch using 6 standard additions of $H_2O_2$ (TraceSelect, Fluka) within the range 10-300 nM to aged (stored at room temperature in the dark for >48 h) seawater (unfiltered).

25    **Macronutrients**

Dissolved macronutrient concentrations (nitrate+nitrite, phosphate, silicic acid; filtered at 0.45 µm upon collection) were measured spectrophotometrically the same day as sample collection (Hansen and Koroleff, 2007). For experiments in Crete, phosphate concentrations were determined using the 'magic' method (Rimmelin and Moutin, 2005). The detection limits for macronutrients thereby inevitably varied slightly between the different mesocosm/microcosm/multistressor experiments

30    (Table 1), however this does not adversely affect the discussion of results herein.

**Carbonate chemistry**

$pH_T$ (except where stated otherwise, 'pH' refers to the total pH scale reported at 25ºC) was measured during the Gran Canaria mesocosm using the spectrophotometric technique of Clayton and Byrne (1993) with m-cresol purple in an automated Sensorlab SP101-SM system using a 25ºC-thermostatted 1 cm flow-cell exactly as per González-Dávila et al., (2016). pH during the MesoPat experiments was measured similarly as per Gran Canaria using m-cresol. During MesoArc/MesoMed experiments pH was measured spectrophotometrically as per Reggiani et al., (2016).

**Biological parameters**

Chlorophyll a was measured by fluorometry as per Welschmeyer (1994). Bacterial production was determined by incorporation of tritium-labelled leucine ($^3$H-Leu) using the centrifugation procedure of Smith and Azam (1992). Conversion of leucine to carbon (C) was done with the theoretical factor 3.1 kg C mol$^{-1}$ leucine. In Gran Canaria, flow cytometry was conducted on 2 mL water samples which were fixed with 1% paraformaldehyde (final concentration), flash frozen in liquid $N_2$ and stored at -80ºC until analysis. Samples were analysed (FACSCalibur, Becton Dickinson) with a 15 mW laser set to excite at 488 nm (Gasol and del Giorgio, 2000). Subsamples (400 µL) for the determination of heterotrophic bacteria were stained with the fluorochrome SybrGreen-I (4 µL) at room temperature for 20 min and run at a flow rate of 16 µL min$^{-1}$. Cells were enumerated in a bivariate plot of 90° light scatter and green fluorescence. Molecular Probes latex beads (1 µm) were used as internal standards. In Crete (MesoMed), the flow cytometry was conducted similarly except for the following minor changes: samples were fixed with 0.5% glutaraldehyde (final concentration), yellow-green microspheres (1 and 10 µm diameter, respectively) were used as internal references during the analysis of bacterial and nanoflagellate populations, and the flow rate was 79-82 µL min$^{-1}$. Subsamples (7-50 L) for zooplankton composition and abundance were preserved in 4% borax buffered formaldehyde solution and analysed microscopically.

## 3.0 Results

### 3.1 $H_2O_2$ time series during outdoor mesocosm incubations; Mediterranean and Gran Canaria

In order to understand the controls on $H_2O_2$ concentrations in incubations, time series of $H_2O_2$ are first presented for those experiments with the highest resolution data. Also of interest are trends in bacterial productivity following the observation that $H_2O_2$ decay constants appear to correlate with bacterial abundance in a range of natural waters (Cooper et al., 1994). The concentration of $H_2O_2$ was followed in all treatments on all sampling days during the Gran Canaria and MesoMed mesocosms. In Gran Canaria, comparing mean (±SD) $H_2O_2$ in all mesocosms across a $pCO_2$ gradient (400-1450 µatm) with $H_2O_2$ in ambient seawater outside the mesocosms, $H_2O_2$ was generally elevated within the mesocosms compared to ambient seawater (Fig. 1). The only exception was a short time period under post-bloom conditions when bacterial abundance peaked and daily integrated light intensity was relatively low (compared to the mean over the duration of the experiment) for 3 consecutive days (experiment days 25-27, Hopwood et al., 2018). No clear trend was observed with respect to the temporal trend in $H_2O_2$ and the $pCO_2$ gradient. $H_2O_2$ concentration in the baseline $pCO_2$ treatment was close to the mean (400-1450 µatm) for the duration of the 28 day experiment.
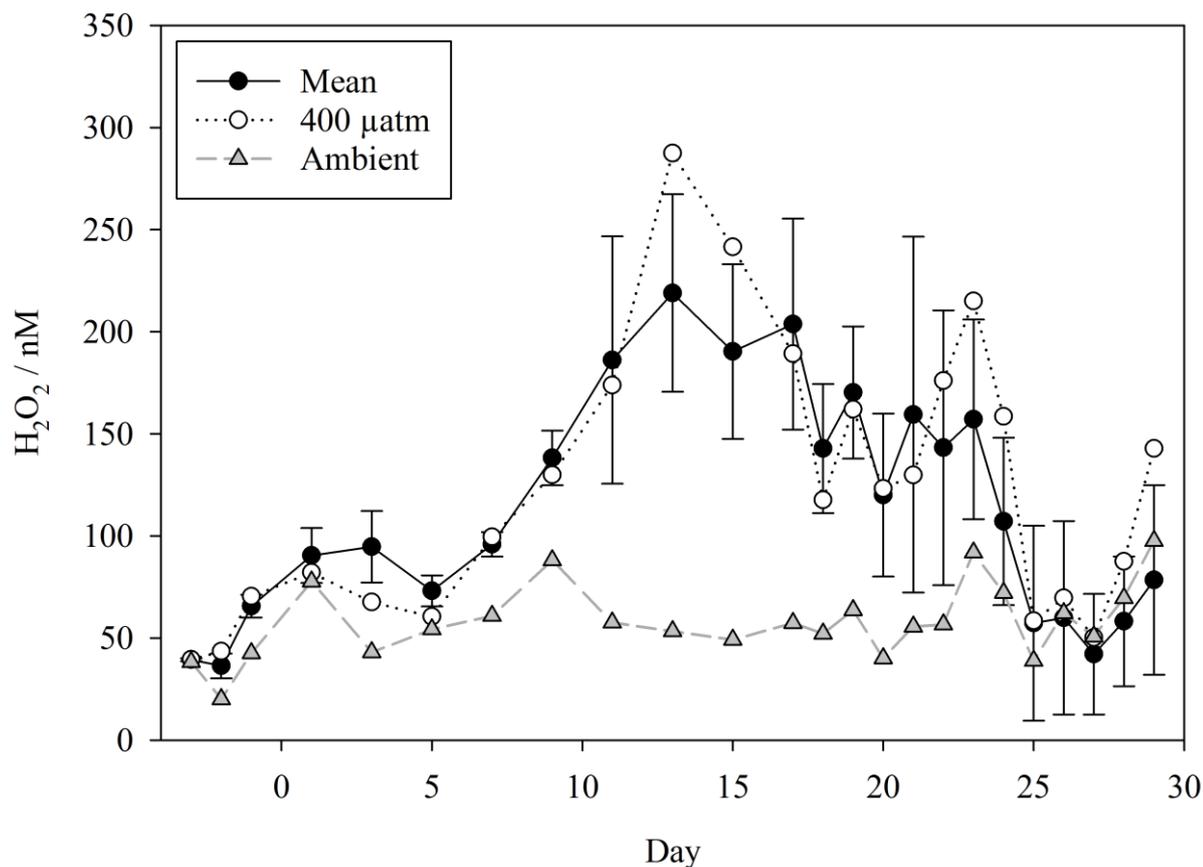
**Figure 1: A summary of $H_2O_2$ over the duration of a $pCO_2$ gradient mesocosm in Gran Canaria. Data from Hopwood et al., (2018). The mean (± SD) mesocosm $H_2O_2$ is contrasted with the concentration in ambient surface seawater immediately outside the mesocosms. In addition to its inclusion in the mean, the baseline 400 µatm $pCO_2$ treatment is shown separately to allow comparison with ambient surface seawater.**

During MesoMed (Fig. 2) an additional mesocosm tank was filled (Tank 11) and maintained without any additions (no macronutrients, no DOC, no zooplankton) alongside the 10 mesocosm containers. As per the Gran Canaria mesocosm, $H_2O_2$ concentrations were also followed in ambient seawater throughout the duration of the MesoMed experiment. The MesoMed mesocosm was however conducted in an outdoor pool facility, so the ambient concentration of $H_2O_2$ in coastal seawater refers to a site approximately 500 m away from the incubation pool. Ambient $H_2O_2$ was generally higher than that observed within the mesocosm with a median concentration of 120 nM around midday (Fig. 2(a)).
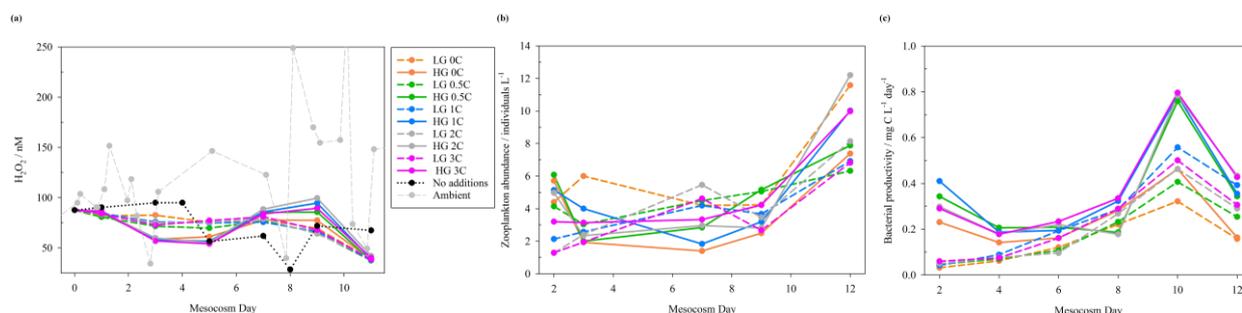
**Figure 2: (a) $H_2O_2$ in all mesocosms during the MesoMed mesocosm in Gouves, Crete. A 10-treatment matrix (as per Fig. S1) was used (b) Zooplankton abundances showed a rapid convergence in the HG/LG status of the mesocosms after day 3 (c) The trend in bacterial productivity showed broad similarity within the HG and LG treatment groups.**

$H_2O_2$ during the MesoMed experiment was relatively constant in terms of the range of concentrations measured over the 11 day duration of the experiment (Fig. 2), especially when compared to the Gran Canaria mesocosm (Fig. 1). A notable clustering of the high ('HG') and low ('LG') zooplankton tanks was clearly observed between days 1 and 9 (Fig. 2) (addition of zooplankton took place immediately after day 1 sampling). $H_2O_2$ concentration in the high zooplankton tanks initially declined more strongly than the low zooplankton tanks, then re-bounded together after day 5 (Fig. 2). This trend closely matched that observed in zooplankton biomass. Dilution experiments to estimate zooplankton grazing and zooplankton abundance (Fig. 2) both suggested that between days 3 and 7, the high/low grazing status of the mesocosms converged i.e. grazing declined in the tanks to which zooplankton had initially been added and increased in the tanks to which no zooplankton had been added such that initial 'high/low' grazing labels became obsolete (Rundt, 2016). $H_2O_2$ concentration declined sharply in all treatments on day 11, except in the no-nutrient addition mesocosm, coinciding with a pronounced increase in zooplankton abundance and occurring just after bacterial productivity peaked in all treatments (Fig. 2).

$H_2O_2$ decay rate constants in the dark (measured using freshly collected seawater at the MesoMed fieldsite over 24 h and assumed to be first order) were 0.049 $h^{-1}$ (unfiltered) and 0.036 $h^{-1}$ (filtered, Satorius 0.2 μm) corresponding to half-lives of 14 h and 19 h, respectively, which are within the range expected for coastal seawater (Petasne and Zika, 1997).

## 3.2 $H_2O_2$ trends during 20 L scale indoor multistressor (Patagonia and Mediterranean) and microcosm (Patagonia) incubations

A sustained decline in $H_2O_2$ concentration was found whenever ambient seawater was moved into controlled temperature rooms with artificial diel light cycles (e.g Fig. 3) which were used to incubate all 20 L scale multistressor and microcosm experiments discussed herein (Table 1). Final $H_2O_2$ concentrations in these 20 L scale experiments were thereby generally low compared to those measured in corresponding ambient surface waters and to the corresponding outdoor experiments in the same locations with natural lighting.
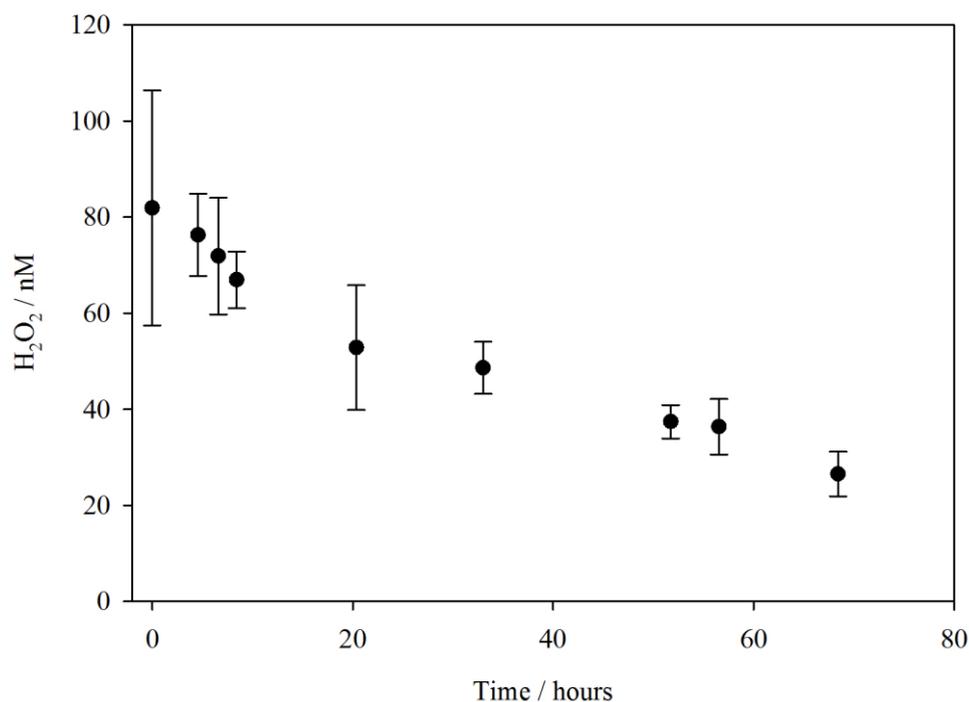
**Figure 3: Seawater from the MesoMed mesocosm (without macronutrient, DOC or zooplankton amendment) was used to fill a 20 L HDPE container which was then incubated under the synthetic lighting used in the Mediterranean multistressor experiment for 72 h with regular sub-sampling for analysis of $H_2O_2$.**
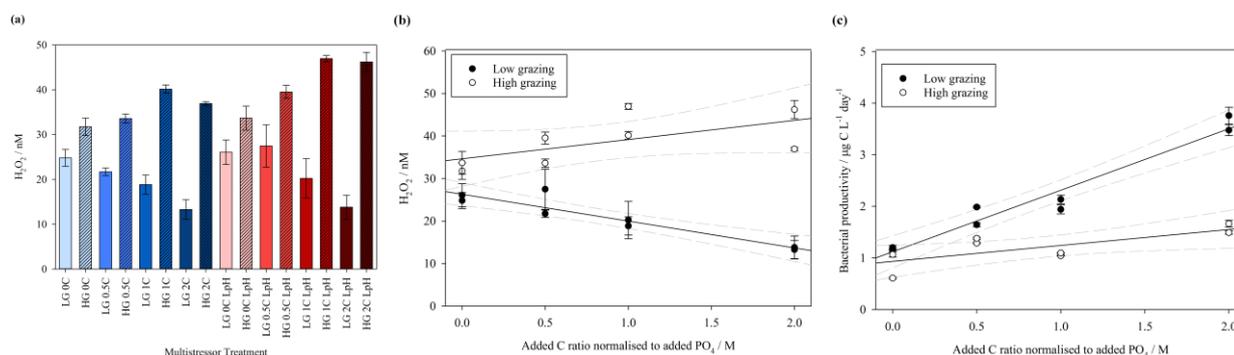
**Figure 4: (a) Multistressor $H_2O_2$ concentrations at the end of the MesoMed multistressor experiment (Day 9). Ambient pH (blue), low pH (red); high grazing (hashed); carbon (C) added at 0, 0.5, 1.0, 1.5 and 2.0 × Redfield carbon: phosphate ratio. (b) Plotting both ambient and low pH datapoints together, which exhibited no statistically significant difference in $H_2O_2$ concentrations, final $H_2O_2$ concentration showed contrasting trends between high and low grazing treatments over the added C gradient. 95% confidence intervals are shown. (c) Bacterial productivity, measured via leucine incorporation, during the same experiments..**

$H_2O_2$ concentrations by the end of the MesoMed multistressor experiments (day 9) were universally low compared to the range found in comparable ambient waters and the outdoor mesocosm incubation conducted at the same fieldsite (Fig. 2). As was the case in the MesoMed mesocosm, a clear difference was noted between $H_2O_2$ concentrations in the high and low

zooplankton addition treatments, with the high grazing always resulting in higher $H_2O_2$ concentrations (Fig. 4 (b)). Any effect of pH was less obvious, with similar results obtained between ambient (initially 8.08 ± 0.02) and low (initially 7.64 ± 0.02) pH treatments (Fig. 4 (a)) and thus low and ambient pH treatments are not distinguished in Fig. 4 (b) and (c). An effect of the imposed C gradient on $H_2O_2$ concentrations was notable in both the high and low grazing treatments, yet the effect

5   operated in the opposite direction (Fig. 4 (b)). In high grazing treatments, increasing C corresponded to increasing extracellular $H_2O_2$ concentrations; whereas in low grazing treatments, increasing C corresponded to decreasing extracellular $H_2O_2$ concentrations. Bacterial productivity increased with added C in both high and low grazing treatments, but there was a more pronounced increase under low grazing conditions (Fig. 4 (c)).

10  At the end of the Patagonia multistressor (day 8), $H_2O_2$ concentrations were similarly low compared to ambient surface waters at the Patagonia fieldsite (Fig. 5 (a)), although there was a greater range of results. In the low pH treatment (initially 7.54 ± 0.09), $H_2O_2$ concentrations were significantly higher (Mann-Whitney Rank Sum test p 0.02) compared to the unmodified pH treatment (initially 8.01 ± 0.02). Contrary to the results from the same experiment in the Mediterranean (Fig. 4), there was no significant difference between high/low grazing treatments (Mann-Whitney Rank Sum test p 0.65).

15  Bacterial productivity also showed similar results between the high and low grazing treatments (Fig. 5 (b)). Data from day 5 (the last day bacterial productivity was measured) showed a similar gradient in increased bacterial productivity with added C for both high/low grazing treatment groups (linear regressions HG 0.64, $R^2$ 0.70 and LG 0.72, $R^2$ 0.92).
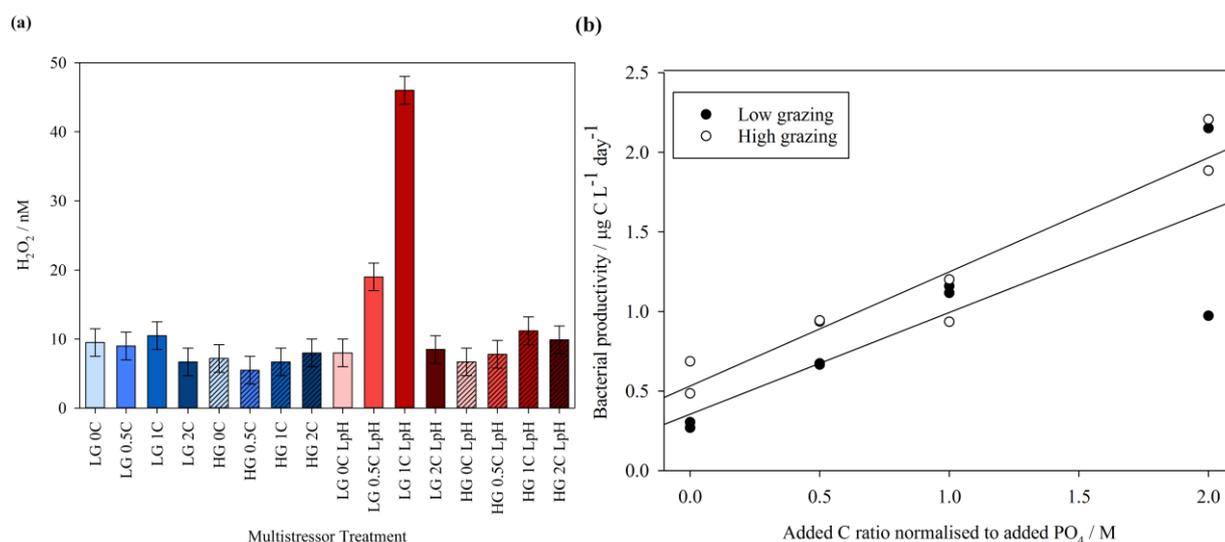
**Figure 5: (a) Multistressor $H_2O_2$ concentrations at the end of the MesoPat multistressor experiment. Normal pH (blue), low pH**
20  **(red); high grazing (hashed); DOC added at 0, 0.5, 1.0, and 2.0 × Redfield carbon (C):phosphate ratio indicated by increasing colour density. (b) Plotting both high and low grazing datapoints together (which exhibited no statistically significant difference in $H_2O_2$ concentrations), bacterial productivity showed similar trends between the HG and LG treatments.**

The Patagonia microcosm experiment, also conducted using 20 L HDPE containers and artificial lighting, yielded no clear trend with respect to $H_2O_2$ concentrations over the imposed C gradient (Fig. 6, day 11), but the high grazing treatments were

associated with higher $H_2O_2$ concentrations (t-test, p 0.017). Bacterial productivity was not systematically different across the high/low grazing treatment groups, nor was there as clear a trend in bacterial productivity with respect to the added C gradient (Fig. 6 (c)) compared to the Patagonia (Fig. 5 (b)) or Mediterranean (Fig. 4 (c)) multistressor experiments.
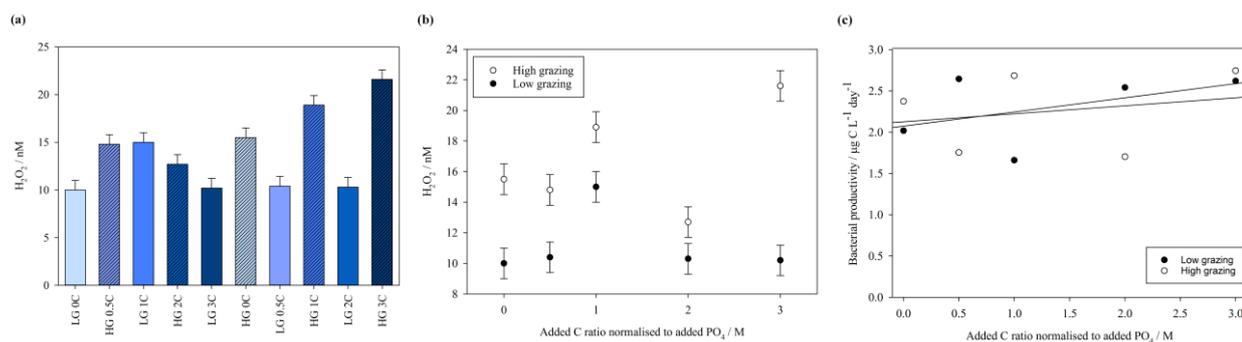
**Figure 6: (a) Microcosm $H_2O_2$ concentrations at the end of the MesoPat microcosm experiment. High grazing treatments are hashed; DOC added at 0, 0.5, 1.0, 2.0 and 3.0 × Redfield carbon (C):phosphate ratio indicated by increasing colour density. (b) No clear trend was evident across the DOC gradient, but high grazing was consistently associated with higher $H_2O_2$ concentration. (c) Bacterial productivity in the same experiment.**

### 3.2 Diurnal cycling of $H_2O_2$; results from the Mediterranean

In addition to the trends observed over the duration of multi-day incubation experiments, a diurnal variability in $H_2O_2$ concentrations is expected. The diurnal cycle of $H_2O_2$ concentrations during MesoMed was followed in the no-addition tank (number 11) over 2 days with markedly different $H_2O_2$ concentrations (Fig. 4). An additional cycle was monitored at a nearby coastal pier (Gouves) for comparative purposes. The mean difference between mid-afternoon and early-morning $H_2O_2$ could also be deduced from discrete time points collected over the experimental duration in seawater close to the pool facility. All time series are plotted against local time (UTC+1). Sunrise/sunset was as follows: (May 15) 06:15, 20:17; (May 19) 06:12, 20:20. All three time series showed the expected peak in $H_2O_2$ concentrations during daylight hours, but the timing of peak $H_2O_2$ concentration and the range of concentrations observed differed between mesocosms and coastal seawater. The intraday range in $H_2O_2$ concentrations in Gouves, and the afternoon peak in $H_2O_2$, (Fig. 7) was similar to that observed previously in Gran Canaria (Hopwood et al., 2018). Yet both the mesocosm diurnal time series exhibited notably limited diurnal ranges and peak $H_2O_2$ concentration occurred earlier, around midday (Fig. 7), than in coastal waters.
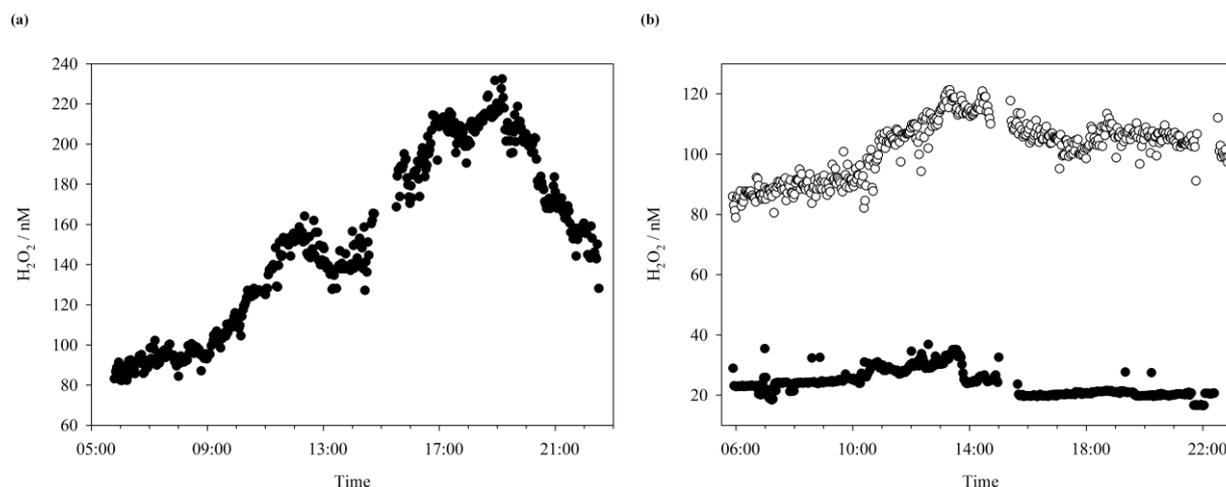
**Figure 7: (a) Diurnal cycling of $H_2O_2$ in coastal seawater (Gouves, Crete 17 May) and (b) in the no addition tank (number 11) during the MesoMed mesocosm on May 15 (open circles) and May 19 (closed circles) 2016 (experiment days 4 and 8, respectively).**

### 3.3  Ancillary experiments to investigate links between microbial groups (bacterial, zooplankton) and extracellular $H_2O_2$

In addition to comparing $H_2O_2$ concentrations in different incubation experiments to assess the effect of experiment setup on extracellular $H_2O_2$ concentrations, potential links between microbial groups and $H_2O_2$ were explored. The Ocean Certain experiments included a high/low zooplankton addition treatment (Table 1). During all Ocean Certain experiments and the Gran Canaria mesocosm (Table 1), data was available on the abundance of bacteria and zooplankton throughout the experiment. We focus on zooplankton because of the top-down control they may exert on primary production and the potential for grazing to release trace species into solution which may affect $H_2O_2$ biogeochemistry. Bacteria were a key focus because of the hypothesis that bacteria are, via the production of peroxidase/catalase enzymes, the main sink for $H_2O_2$ in surface aquatic environments (Cooper et al., 1994).

Over a 20 h incubation (4 h darkness, 16 h light) in an experiment with varying concentrations of copepods (0-25 $L^{-1}$) grazing on an intermediate density of a diatom (initially 3 µg $L^{-1}$ chlorophyll a), $H_2O_2$ concentrations showed no inter-treatment differences (Fig. 8). A diatom was selected as phytoplankton stock because cell normalized $H_2O_2$ production rates for diatoms appear to be generally at the low end of the observed range for phytoplankton groups (Schneider et al., 2016). Fe(II) concentration (measured at the same time as per 'Part B') also appeared to be unaffected by the copepod density as the difference between treatments was almost negligible (<0.04 nM).
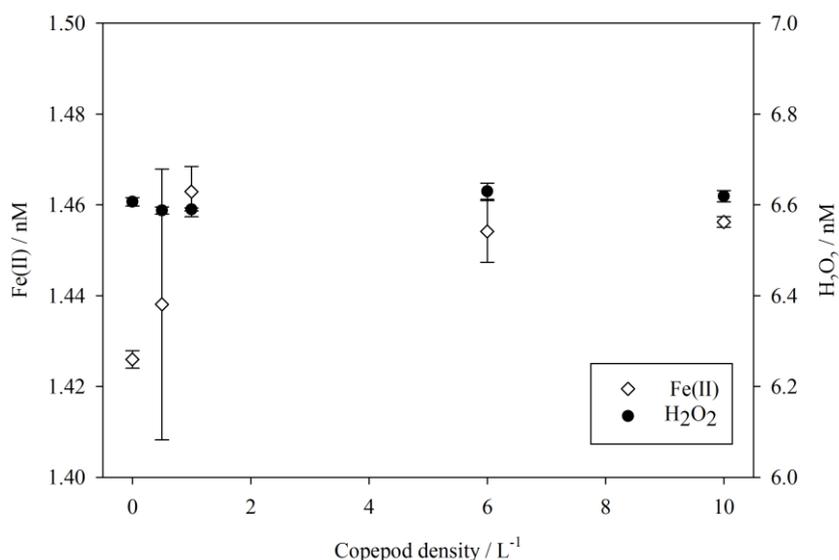
**Figure 8: H$_2$O$_2$ and Fe(II) concentrations in a culture of diatoms growing in coastal seawater after 20 h of incubation with a zooplankton gradient imposed by addition of copepods.**

At the end of the Mediterranean mesocosm, seawater (extracted from the baseline treatment from the mesocosm on day 11) was used in two side experiments. During both the extracellular H$_2$O$_2$ concentration was manipulated, with each treatment triplicated. In all cases the mean (±SD) of three replicate treatments is reported. The high-medium-low H$_2$O$_2$ concentration gradient used in each experiment was determined by considering the ambient concentration of H$_2$O$_2$ in the mesocosms (e.g. Fig. 2) and in ambient seawater close to the mesocosm facility. After the first daily H$_2$O$_2$ measurements were made, the required spikes to maintain the desired H$_2$O$_2$ gradient were calculated based on measured rates of H$_2$O$_2$ decay. H$_2$O$_2$ and catalase spikes were then added at sunset followed by gentle mixing.

A test specifically to investigate the effect of the multistressor/microcosm experimental set up on bacterial activity was conducted in 500 mL trace metal clean LDPE bottles under the artificial lighting conditions (~80 µmol quanta m$^{-2}$ s$^{-2}$) used for the Mediterranean microcosm. H$_2$O$_2$ concentrations again verified that manipulation with H$_2$O$_2$ spikes successfully created a high, medium and low H$_2$O$_2$ treatment (mean for triplicate low/medium/high treatments: 40 ± 2, 120 ± 6, 230 ± 7 nM H$_2$O$_2$). Bacterial production showed no statistically significant (ANOVA, P 0.562) difference between low, medium and high H$_2$O$_2$ treatments.

For a concurrent manipulation in the Mediterranean using 20 L HDPE containers incubated outdoors, a gradient in H$_2$O$_2$ concentrations was similarly imposed. These manipulations successfully produced a clear gradient of H$_2$O$_2$ conditions with relatively consistent H$_2$O$_2$ concentrations within each triplicated set (Fig. 9 (a)). After day 5 no further manipulations were

conducted and $H_2O_2$ accordingly began to converge towards the medium (no $H_2O_2$ spike, no active catalase spike) treatment. Flow cytometry, conducted on low/medium/high samples at $8 \times 24$ h intervals over the experiment duration, measured no significant (ANOVA, $p < 0.05$) difference between the 3 treatments for cell counts of any group (bacteria are shown as an example, Fig. 9 (b)).
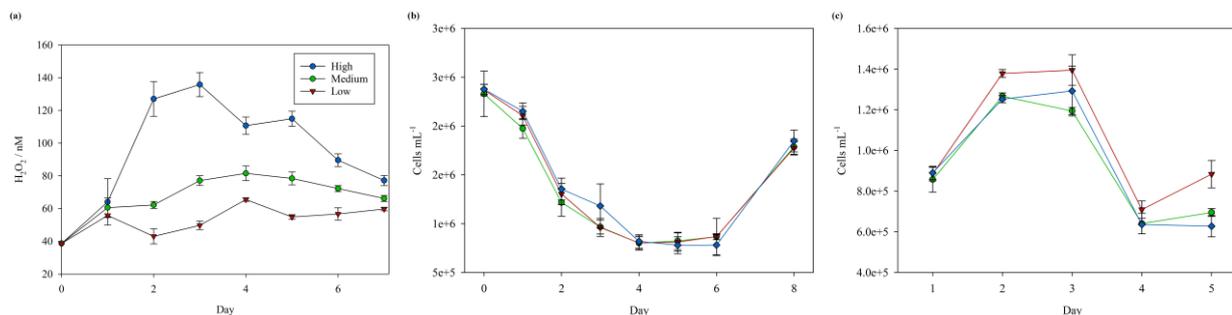
**Figure 9: (a) $H_2O_2$ gradient during the 20 L scale Mediterranean side experiment where a $H_2O_2$ gradient was created with $H_2O_2$ spikes and catalase (b) bacteria abundance during the same Mediterranean experiment (c) bacteria abundance for a similar incubation in Gran Canaria. Mean and standard deviations of triplicate treatments are plotted in all cases.**

10 A similar side experiment was conducted in Gran Canaria, but one critical difference was the addition of macronutrients at the start of the experiment, as per the mesocosm at the same location (Table 1). Measurement of $H_2O_2$ concentrations, which were initially $43 \pm 1$ nM (mean of all $3 \times 3$ replicates at day 0), confirmed that a gradient was maintained over the 5-day duration of the experiment (mean $210 \pm 113$, $62 \pm 14$ and $47 \pm 8$ nM in the high, medium and low $H_2O_2$ treatments, respectively). Some modest shifts in phytoplankton group abundance were observed over the duration of this experiment in

15 response to a similar low/medium/high $H_2O_2$ gradient. Slightly higher cell counts of bacteria were consistently observed in the low $H_2O_2$ treatment relative to the medium and high $H_2O_2$ treatment (Fig. 9 (c)). Only the difference between the low and medium/high treatments was significant (ANOVA, p 0.028)- no significant difference was found between the medium and high $H_2O_2$ treatments (ANOVA, p 0.81).

## 4 Discussion

20 **4.1 Bacteria, zooplankton and extracellular $H_2O_2$ trends**
Throughout, no clear effect was evident of changing pH on $H_2O_2$ concentrations. The 440-1450 µatm $pCO_2$ gradient applied in Gran Canaria, which corresponded to a pH range of approximately 7.5-8.1, and the contrasting ambient/low pH (a reduction in pH of 0.4-0.5 from ambient waters was imposed) applied during both the Ocean Certain mesocosms and microcosm incubations (Table 1) exhibited no obvious change in equilibrium extracellular $H_2O_2$ concentration. Similarly no

25 change was evident in Gran Canaria when contrasting the diurnal cycling of $H_2O_2$ in the 400 and 1450 µatm $pCO_2$ treatments (Hopwood et al., 2018). In the incubation experiments, whenever there was a sustained difference in extracellular $H_2O_2$ concentrations between treatment groups (MesoMed Fig. 2 and Mediterranean multistressor Fig. 4), the main difference

arose between 'high' and 'low' zooplankton addition treatments. However, determining the underlying reason for this was complicated by the shifts in zooplankton abundance during the experiments (e.g. Fig. 2 (b)).

In the Patagonian multistressor (Fig. 5) and microcosm (Fig. 6) incubations no significant effect of increased zooplankton abundance was apparent on extracellular $H_2O_2$. Two reasons for this can be considered. First, in Patagonia the initial ratio of zooplankton between the high and low treatments was the smallest of the Ocean Certain experiments (17:14, see Table 1B) and thus a large difference might not have been anticipated compared to the experiments where this initial ratio was always considerably higher. However, the mean ratio of HG:LG zooplankton by the end of the Patagonian multistressor had increased to 9:5. By comparison, during MesoMed (when the HG:LG zooplankton abundance converged during the experiment, Fig. 2(b)) the HG:LG ratio after day 1 varied within the range 0.32-1.6 and thus the final ratio of 1.8 in the Patagonian multistressor was not particularly low. A more distinct difference however arose in bacterial productivity (Fig. 5 (b)). Unlike MesoMed, the Patagonian multistressor and microcosm incubations showed little difference in bacterial productivity between the high and low grazing treatments. Thus the effects of zooplankton with respect to shifts in the abundance of other microbial groups (rather than grazing itself) may be the underlying reason why extracellular $H_2O_2$ concentrations sometimes, but not consistently, changed between high and low grazing treatments. Second, in any case $H_2O_2$ concentrations at the end of the Patagonian experiments were also very low (almost universally <20 nM) and thus the signal:noise ratio unfavourable for detecting differences between treatments.

Furthermore, the effect of higher zooplankton populations was not a consistent positive/negative change in extracellular $H_2O_2$. During the post-nutrient addition phase in Gran Canaria, the single treatment with slower nutrient drawdown (mesocosm 7) due to high grazing pressure exhibited relatively high $H_2O_2$ (Hopwood et al., 2018). During MesoMed, increases in zooplankton abundance coincided with decreases in $H_2O_2$ concentration (Fig. 2). Similarly, during the 16 treatment incubation conducted in Crete (Fig. 4), the effect of adding zooplankton was the same; high zooplankton treatments exhibited low $H_2O_2$ concentration. As high zooplankton are correlated during some experiments, and anti-correlated in others, with $H_2O_2$, the underlying cause did not appear to be that $H_2O_2$ is generally produced by the process of grazing (i.e. as a by-product of feeding). Further support for this argument was found in the results of a simple side experiment adding copepods (*Calanus finmarchichus*) to a diatom culture (*Skeletonema costatum*) (Fig. 8). No measurable change in extracellular $H_2O_2$ concentration was found at higher densities of copepods either during a 16 h light incubation, or after 4 h of incubation in the dark (Fig. 8). There are two obvious limitations in this experiment; a different result may have been obtained with a different combination of copepod and phytoplankton, and standard f/2 medium contains the ligand ethylenediaminetetraacetic acid (EDTA) which may affect $H_2O_2$ formation rates by complexing trace species involved in $H_2O_2$ cycling (e.g. dissolved Fe and Cu). Nonetheless, it is known that cellular ROS production rates vary at the species level (Schneider et al., 2016; Cho et al., 2017), so shifts in species composition as a result of zooplankton addition are a plausible underlying cause of changes in extracellular $H_2O_2$ concentration. We summarise that any correlation between $H_2O_2$ and

zooplankton thereby appears to have arisen from the resulting change in the abundance of microbial species, and thus the net contribution of biota to extracellular $H_2O_2$ concentration, rather than from the act of grazing itself.

Combing all available $H_2O_2$ concentrations for which corresponding bacterial abundance is available (Fig. 10) from all
5    experiments (except the side experiments where $H_2O_2$ was manipulated using catalase or $H_2O_2$ spikes), provides some evidence for the dominance of bacteria as a $H_2O_2$ sink (Cooper et al., 1994). Whilst the correlation between extracellular $H_2O_2$ and bacteria cell counts was much stronger in some experiments than others ($R^2$ from 0.09-0.55), there was a notable absence of high-$H_2O_2$, high-bacteria datapoints in any experiment (Fig. 10). The observed distribution is consistent with a scenario where bacteria dominate $H_2O_2$ removal, but other factors (possibly including experiment design) can also lead to
10   low $H_2O_2$ conditions independently of bacterial abundance.
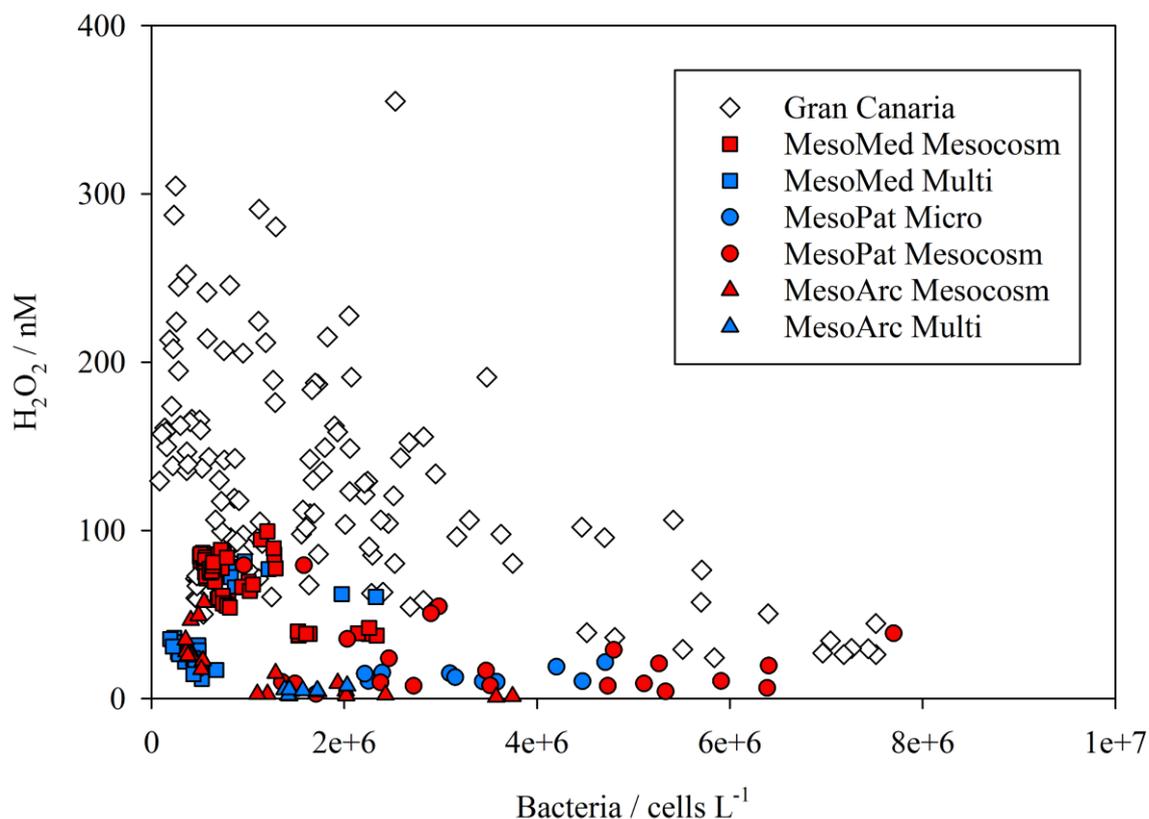
**Figure 10: Bacterial cell counts and $H_2O_2$ for all available data from all incubation experiment time-points where both measurements were made within 24 h of each other.**

**4.2 Changes in extracellular $H_2O_2$ due to experiment design**

15   When all available $H_2O_2$ datapoints were normalized to ambient $H_2O_2$ at the respective fieldsite, which varied between our locations (Table 2), some qualitative inter-experiment trends were evident. Experiments incubated with artificial lighting

generally exhibited the lowest concentrations, while higher normalized $H_2O_2$ concentrations were observed in the closed

HDPE mesocosms (MesoMed, MesoPat, MesoArc) and then the open Gran Canaria mesocosm experiment (Fig. 11 (b) and

(c)).

| Location | Season | Latitude | Salinity | Temperature / °C | $H_2O_2$ / nM |
|---|---|---|---|---|---|
| Taliarte, Gran Canaria | March 2016 | 30.0° N | 36.6-36.8 | 18-19 | 10-50 |
| Gouves, Crete | May 2016 | 35.3° N | NA | 19-20[a] | 34-410[b] |
| Comau fjord, Patagonia | November 2014 | 42.4° S | 3.9-12.8 | 9.7-13 | 120-680 |
| Kongsfjorden, Svalbvard | July 2015 | 78.9° N | 9.0-35.2 | 5.0-9.0 | 10-100 |

**Table 2. Range of water properties in coastal seawater adjacent to mesocosms. 'NA' not applicable. [a] Temperature of pool facility at HCMR, [b] Coastal seawater approximately 500 m from HCMR facility.**
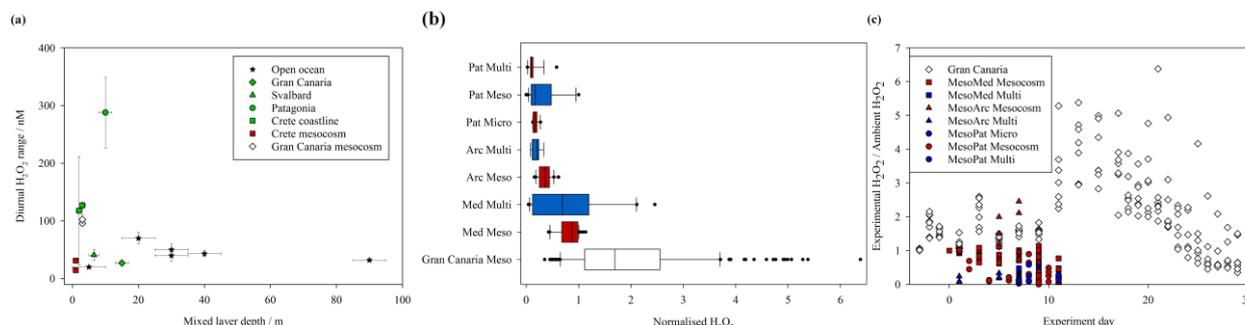
**Figure 11: (a) Observed diurnal ranges in $H_2O_2$ concentrations. Black stars show literature surface marine values and green shapes in-situ experiments corresponding to experiment field site locations (b) $H_2O_2$ across all experiments as a fraction of ambient $H_2O_2$. For the Ocean Certain fieldsites (Mediterranean, Arctic and Patagonia) red shapes are outdoor mesocosms and blue shapes indoor incubations. Median, 10th/25th/75th/90th percentiles and all outliers are shown. (c) $H_2O_2$ time series across all experiments normalised as per (b).**

During all periods when high resolution $H_2O_2$ time series were obtained, a clear diurnal trend was observed with a peak in

$H_2O_2$ concentration occurring around midday (Fig. 7). Yet the range of concentrations within the two MesoMed diurnal

experiments (31.2 ± 2.3 nM and 14.5 ± 2.7 nM) was limited compared to those observed previously within a Gran Canaria

mesocosm (96 ± 4 and 103 ± 8 nM, Hopwood et al., 2018). For comparison, the diurnal ranges reported in surface waters of

the Atlantic, Gulf of Mexico and sub-tropical equatorial Pacific are 20-30 nM (Yuan and Shiller, 2001), 40-70 nM (Zika et

al., 1985) and 40 nM,[1] respectively with no clear systematic trend associated with changes in mixed layer depth (Fig. 11 (a)).

Within mesocosms and the coastal mesocosm fieldsites, the range was more variable. Notably, the MesoMed mesocosm

diurnal ranges (15 and 31 nM) were considerably lower than that observed at two corresponding coastal sites (one monitored

over a single diurnal cycle, 127 ± 5 nM; one at regular intervals over the duration of the experiment, 118 ± 94 nM). Whereas,

conversely, for the Gran Canaria mesocosm the ~100 nM diurnal range was much greater than that observed (27.0 ± 3.1 nM)

in ambient surface waters (Fig. 11 (a)).

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[1] Unpublished data kindly provided by Insa Rapp (GEOMAR).

There are inevitably limits to what can be determined from contrasting available data on $H_2O_2$ concentration from multiple incubation experiments due to the different experiment designs (see Table 1). Yet the experiment setup with respect to moderating light during an experiment appears to be critical to establishing the equilibrium $H_2O_2$ concentration and can either enhance or retard the extracellular concentration of $H_2O_2$ during the experiment. The diurnal range plotted for all mesocosm experiments reflected increased $H_2O_2$ concentrations during daylight hours. This concentration range was suppressed in the closed HDPE containers (e.g. Mediterranean), yet enhanced in open polyurethane bags (Gran Canaria). During the Ocean Certain multistressor and microcosm experiments, incubated indoors in 20 L HDPE containers, the diurnal range in $H_2O_2$ concentrations was suppressed sufficiently that no increase in $H_2O_2$ was apparent during simulated daylight hours. Lighting conditions for the experiments therefore could explain both the contrasting change in the diurnal range of $H_2O_2$ (Fig. 11a), and the shift in the gradient between bacteria and $H_2O_2$ under different experiment conditions (Fig. 10).

### 4.3 ROS, bacteria and the Black Queen Hypothesis

Results from experiments where $H_2O_2$ concentrations were manipulated were mixed. In Crete, there was no evidence of strong positive or negative effects of $H_2O_2$ concentrations on any specific microbial group (Fig. 9). In Gran Canaria, under different experimental conditions (macronutrients were added, whereas in Crete the experiment was run without a macronutrient spike), a small increase in bacterial abundance was found at low $H_2O_2$ concentrations (+27%, Fig. 9 (c))). This result alone should be interpreted with caution, as the addition of catalase can have other effects in addition to lowering $H_2O_2$ concentration (Morris, 2011), yet it is intriguing to consider the role of $H_2O_2$ as an intermediate in the cycling of DOM alongside the role of bacteria as the dominant $H_2O_2$ sink.

Photochemistry both enhances the lability of DOM (Bertilsson and Tranvik, 1998; Keiber et al., 1990) (thus making it more bioavailable as a substrate for bacteria) and causes the direct photochemical oxidation of DOM into dissolved inorganic carbon (Miller and Zepp, 1995; Granéli et al., 1996) (thus rendering it unavailable as a substrate for bacteria). ROS may enhance both of these processes, but few attempts have been made to determine the effect of manipulating ROS concentrations on photochemical DOM degradation rates, especially in the marine environment (Pullin et al., 2004). Yet in experiments using furfuryl alcohol to suppress ROS in lake water, the rate of dissolved inorganic carbon formation when exposed to light decreased 20% and bacterial populations when later incubated in this ROS-quenched water were 4-fold higher than water with 'normal' ROS activity (Scully et al., 2003) implying that ROS removal was beneficial for bacteria. The results of experiments conducted in freshwater environments are not directly applicable to the marine environment due to the different conditions in the ambient water column, but it is plausible that a similar mechanism underpinned the increase in bacteria abundance observed in Gran Canaria following the artificial lowering of $H_2O_2$ concentrations (Fig. 9). A large difference in bacterial populations between the presence and absence of some ROS species (Scully et al., 2003) raises interest in how important an influence changes in ROS concentration could be on the availability of DOM for bacterial

productivity in the surface marine environment. If heterotrophic bacteria are the dominant $H_2O_2$ sink (Cooper et al., 1994), which the observed trend between bacterial abundance and extracellular $H_2O_2$ across a broad range of incubation experiments is consistent with (Fig. 10), this is also interesting in light of the Black Queen Hypothesis. BQH (Morris et al., 2012) assumes that the sole major benefit of producing enzymes that remove extracellular $H_2O_2$ is protection against the

5 oxidative stress associated with high $H_2O_2$ concentrations- which is a communal benefit (Zinser, 2018). However, if increasing extracellular $H_2O_2$ concentrations accelerate the degradation of labile DOM to dissolved inorganic carbon, a second benefit of $H_2O_2$ removal is the enhanced availability of this DOM to heterotrophs. Thus it could possibly be more favourable for heterotrophic species to maintain genes associated with the removal of $H_2O_2$ than autotrophic species because, in addition to the shared communal benefit of lowering oxidative stress, heterotrophs would suppositionally benefit more

10 directly than autotrophs from the enhanced stability of labile DOM under low $H_2O_2$ conditions.

## 5    Conclusions

Extracellular $H_2O_2$ concentrations and bacterial abundances over a broad range of incubation experiments conducted in the marine environment support the hypothesis that bacterially produced enzymes are the dominant $H_2O_2$ sink. If heterotrophic bacteria are generally the main sink for $H_2O_2$ in surface marine environments, it is of interest to determine whether changes

15 in extracellular $H_2O_2$ concentration measurably affect the photochemical transformation of DOM transformation to dissolved inorganic carbon. If increasing equilibrium ROS concentrations decreases the availability of labile DOM as a substrate for heterotrophs, this may affect which group/species produce catalase/peroxidase enzymes.

It was also apparent from comparing multiple experiments that incubation experiment design is also a strong influence on

20 $H_2O_2$ concentrations. Closed HDPE mesocosms exhibited concentrations 10-90% lower than those expected in the corresponding ambient seawater, whereas an open (lidless) mesocosm exhibited concentrations 2-6 fold higher than ambient seawater. The diurnal range in $H_2O_2$ within incubations was also correspondingly increased in experiments where $H_2O_2$ concentration was artificially high, and vice-versa where $H_2O_2$ concentration was artificially low, suggesting enhanced, or reduced, photochemical stress over the diurnal cycle. Incubated experiments thus poorly mimic the biogeochemistry of

25 reactive photo-chemically formed trace species.

## 4    Author Contributions

MH, DP, JG, EA, DT and MA designed the study. MH, NS, DP, ØL, JG, MA, JA, SB, YH, IK, TK and TT undertook work at one or more of the mesocosm/microcosm/multistressor experiments. MH, NS, DP, ØL, JG, JA, LB, SB, YH, TK, IS and TT conducted analytical work. MH, NS, DP, SB and TT interpreted the data. MH coordinated the writing of the manuscript

30 with input from other authors.

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5    603773) is gratefully acknowledged. JA was supported by a Helmholtz International Fellow Award, 2015 (Helmholtz Association, Germany).

## 6    References

Angel, L. D., Fiedler, U., Eden, N., Kress, N., Adelung, D. and Herut, B.: Catalase activity in macro- and microorganisms as an indicator of biotic stress in coastal waters of the eastern Mediterranean Sea, Helgol. Mar. Res., 53(3), 209–218,
10    doi:10.1007/s101520050025, 1999.

Van Baalen, C. and Marler, J. E.: Occurrence of hydrogen peroxide in sea water, Nature, 211(5052), 951, doi:10.1038/211951a0, 1966.

Baltar, F., Reinthaler, T., Herndl, G. J. and Pinhassi, J.: Major Effect of Hydrogen Peroxide on Bacterioplankton Metabolism in the Northeast Atlantic, PLoS One, 8(4), e61051, doi:10.1371/journal.pone.0061051, 2013.

15    Bertilsson, S. and Tranvik, L. J.: Photochemically produced carboxylic acids as substrates for freshwater bacterioplankton, Limnol. Oceanogr., 43(5), 885–895, doi:10.4319/lo.1998.43.5.0885, 1998.

Bogosian, G., Aardema, N. D., Bourneuf, E. V., Morris, P. J. L. and O'Neil, J. P.: Recovery of hydrogen peroxide-sensitive culturable cells of Vibrio vulnificus gives the appearance of resuscitation from a viable but nonculturable state, J. Bacteriol., 182(18), 5070–5075, doi:10.1128/JB.182.18.5070-5075.2000, 2000.

20    Cho, K., Kasaoka, T., Ueno, M., Basti, L., Yamasaki, Y., Kim, D. and Oda, T.: Haemolytic activity and reactive oxygen species production of four harmful algal bloom species, Eur. J. Phycol., 52(3), 311–319, doi:10.1080/09670262.2017.1286525, 2017.

Clayton, T. D. and Byrne, R. H.: Spectrophotometric seawater pH measurements: total hydrogen ion concentration scale calibration of m-cresol purple and at-sea results, Deep Sea Res. Part I Oceanogr. Res. Pap., 40(10), 2115–2129,
25    doi:10.1016/0967-0637(93)90048-8, 1993.

Cooper, W. J., Zika, R. G., Petasne, R. G. and Plane, J. M. C.: Photochemical formation of hydrogen peroxide in natural waters exposed to sunlight, Environ. Sci. Technol., 22(10), 1156–1160, doi:10.1021/es00175a004, 1988.

Cooper, W. J., Shao, C. W., Lean, D., Gordon, A. and Scully, F. E.: Factors affecting the distribution of H2O2 in surface waters, in Environmental Chemistry of Lakes and Reservoirs, vol. 237, pp. 391–422., 1994.

30    Gasol, J. M. and del Giorgio, P. A.: Using flow cytomery for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities., Sci. Mar., 64, 197 – 224, doi:10.3989/scimar.2000.64n2197, 2000.

Gerringa, L. J. A., Rijkenberg, M. J. A., Timmermans, R. and Buma, A. G. J.: The influence of solar ultraviolet radiation on the photochemical production of H2O2 in the equatorial Atlantic Ocean, J. Sea Res., 51(1), 3–10,

doi:10.1016/j.seares.2003.03.002, 2004.

González-Dávila, M., Santana-Casiano, J. M., Petihakis, G., Ntoumas, M., Suárez de Tangil, M. and Krasakopoulou, E.: Seasonal pH variability in the Saronikos Gulf: A year-study using a new photometric pH sensor, J. Mar. Syst., 162(Supplement C), 37–46, doi:10.1016/j.jmarsys.2016.03.007, 2016.

5   Granéli, W., Lindell, M. and Tranvik, L. J.: Photo-oxidative production of dissolved inorganic carbon in lakes of different humic content, Limnol. Oceanogr., 41(4), 698–706, doi:10.4319/lo.1996.41.4.0698, 1996.

Guillard, R. R. L. and Ryther, J. H.: Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (cleve) Gran., Can. J. Microbiol., 8(2), 229–239, doi:10.1139/m62-029, 1962.

Hansel, C. M., Ferdelman, T. G. and Tebo, B. M.: Cryptic cross-linkages among biogeochemical cycles: Novel insights from
10  reactive intermediates, Elements, 11(6), 409–414, doi:10.2113/gselements.11.6.409, 2015.

Hansen, H. P. and Koroleff, F.: Determination of nutrients, in Methods of Seawater Analysis: Third, Completely Revised and Extended Edition, pp. 159–228., 2007.

Hopwood, M. J., Rapp, I., Schlosser, C. and Achterberg, E. P.: Hydrogen peroxide in deep waters from the Mediterranean Sea, South Atlantic and South Pacific Oceans, Sci. Rep., 7, 43436, doi:10.1038/srep43436, 2017.

15  Hopwood, M. J., Riebesell, U., Arístegui, J., Ludwig, A., Achterberg, E. P. and Hernández, N.: Photochemical vs. Bacterial Control of H2O2 Concentration Across a pCO2 Gradient Mesocosm Experiment in the Subtropical North Atlantic, Front. Mar. Sci., 5, 105, doi:10.3389/fmars.2018.00105, 2018.

Hughes, C. and Sun, S.: Light and brominating activity in two species of marine diatom, Mar. Chem., 181, 1–9, doi:10.1016/j.marchem.2016.02.003, 2016.

20  Keiber, R. J., Zhou, X. and Mopper, K.: Formation of carbonyl compounds from UV-induced photodegradation of humic substances in natural waters: Fate of riverine carbon in the sea, Limnol. Oceanogr., 35(7), 1503–1515, doi:10.4319/lo.1990.35.7.1503, 1990.

Larsen, A., Egge, J. K., Nejstgaard, J. C., Di Capua, I., Thyrhaug, R., Bratbak, G. and Thingstad, T. F.: Contrasting response to nutrient manipulation in Arctic mesocosms are reproduced by a minimum microbial food web model., Limnol. Oceanogr.,
25  60(2), 360–374, doi:10.1002/lno.10025, 2015.

Miller, W. L. and Kester, D. R.: Peroxide variations in the Sargasso Sea, Mar. Chem., 48(1), 17–29, doi:10.1016/0304-4203(94)90059-0, 1994.

Miller, W. L. and Zepp, R. G.: Photochemical production of dissolved inorganic carbon from terrestrial organic matter: Significance to the oceanic organic carbon cycle, Geophys. Res. Lett., 22(4), 417, doi:10.1029/94GL03344, 1995.

30  Moffett, J. W. and Zafiriou, O. C.: An investigation of hydrogen peroxide chemistry in surface waters of Vineyard Sound with H218O2 and 18O2, Limnol. Oceanogr., 35(6), 1221–1229, doi:10.4319/lo.1990.35.6.1221, 1990.

Moffett, J. W. and Zika, R. G.: Reaction kinetics of hydrogen peroxide with copper and iron in seawater, Environ. Sci. Technol., 21(8), 804–810, doi:10.1021/es00162a012, 1987.

Moore, C. a., Farmer, C. T. and Zika, R. G.: Influence of the Orinoco River on hydrogen peroxide distribution and

production in the eastern Caribbean, J. Geophys. Res., 98(C2), 2289, doi:10.1029/92JC02767, 1993.

Morris, J. and Zinser, E. R.: Continuous hydrogen peroxide production by organic buffers in phytoplankton culture media, J. Phycol., 49(6), 1223–1228, doi:10.1111/jpy.12123, 2013.

Morris, J. J.: The "Helper" Phenotype: A Symbiotic Interaction Between Prochlorococcus and Hydrogen Peroxide Scavenging Microorganisms, University of Tennessee., 2011.

Morris, J. J., Johnson, Z. I., Szul, M. J., Keller, M. and Zinser, E. R.: Dependence of the cyanobacterium Prochlorococcus on hydrogen peroxide scavenging microbes for growth at the ocean's surface, PLoS One, 6(2), e16805, doi:10.1371/journal.pone.0016805, 2011.

Morris, J. J., Lenski, R. E. and Zinser, E. R.: The black queen hypothesis: Evolution of dependencies through adaptive gene loss, MBio, 3(2), doi:10.1128/mBio.00036-12, 2012.

Morris, J. J., Johnson, Z. I., Wilhelm, S. W. and Zinser, E. R.: Diel regulation of hydrogen peroxide defenses by open ocean microbial communities, J. Plankton Res., 38(4), 1103–1114, doi:10.1093/plankt/fbw016, 2016.

Petasne, R. G. and Zika, R. G.: Hydrogen peroxide lifetimes in South Florida coastal and offshore waters, Mar. Chem., 56(3–4), 215–225, doi:10.1016/S0304-4203(96)00072-2, 1997.

Price, D., Mantoura, R. F. C. and Worsfold, P. J.: Shipboard determination of hydrogen peroxide in the western Mediterranean sea using flow injection with chemiluminescence detection, Anal. Chim. Acta, 377(2–3), 145–155, doi:10.1016/S0003-2670(98)00621-7, 1998.

Pullin, M. J., Bertilsson, S., Goldstone, J. V. and Voelker, B. M.: Effects of sunlight and hydroxyl radical on dissolved organic matter: Bacterial growth efficiency and production of carboxylic acids and other substrates, Limnol. Oceanogr., 49(6), 2011–2022, doi:10.4319/lo.2004.49.6.2011, 2004.

Redfield, A. C.: On the proportions of organic derivations in sea water and their relation to the composition of plankton, in James Johnstone Memorial Volume, edited by R. J. Daniel, pp. 177–192, University Press of Liverpool., 1934.

Reggiani, E. R., King, A. L., Norli, M., Jaccard, P., Sørensen, K. and Bellerby, R. G. J.: FerryBox-assisted monitoring of mixed layer pH in the Norwegian Coastal Current, J. Mar. Syst., 162, 29–36, doi:10.1016/j.jmarsys.2016.03.017, 2016.

Riebesell, U., Czerny, J., Von Bröckel, K., Boxhammer, T., Büdenbender, J., Deckelnick, M., Fischer, M., Hoffmann, D., Krug, S. A., Lentz, U., Ludwig, A., Muche, R. and Schulz, K. G.: Technical Note: A mobile sea-going mesocosm system - New opportunities for ocean change research, Biogeosciences, 10(3), 1835–1847, doi:10.5194/bg-10-1835-2013, 2013.

Rimmelin, P. and Moutin, T.: Re-examination of the MAGIC method to determine low orthophosphate concentration in seawater, Anal. Chim. Acta, 548(1), 174–182, doi:10.1016/j.aca.2005.05.071, 2005.

Rundt, C.: Organic Carbon Enrichment of Mediterranean Waters: Effects on the Pelagic Microbial Food Web with Emphasis on Microzooplankton Grazing., University of Bremen., 2016.

Schneider, R. J., Roe, K. L., Hansel, C. M. and Voelker, B. M.: Species-Level Variability in Extracellular Production Rates of Reactive Oxygen Species by Diatoms, Front. Chem., 4, 5, doi:10.3389/fchem.2016.00005, 2016.

Scully, N. M., Cooper, W. J. and Tranvik, L. J.: Photochemical effects on microbial activity in natural waters: The

interaction of reactive oxygen species and dissolved organic matter, in FEMS Microbiology Ecology, vol. 46, pp. 353–357., 2003.

Smith, D. C. and Azam, F.: A simple, economical method for measuring bacterial protein synthesis rates in seawater using 3H-leucine, Mar. Microb. Food Webs, 6(2), 107–114, 1992.

5   Voelker, B. M. and Sulzberger, B.: Effects of Fulvic Acid on Fe(II) Oxidation by Hydrogen Peroxide, Environ. Sci. Technol., 30(4), 1106–1114, doi:10.1021/es9502132, 1996.

Welschmeyer, N. A.: Fluorometric analysis of chlorophyll a in the presence of chlorophyll b and pheopigments, Limnol. Oceanogr., 39(8), 1985–1992, doi:10.4319/lo.1994.39.8.1985, 1994.

Yuan, J. and Shiller, A.: The distribution of hydrogen peroxide in the southern and central Atlantic ocean, Deep Sea Res.
10  Part II Top. Stud. Oceanogr., 48(13), 2947–2970, doi:10.1016/S0967-0645(01)00026-1, 2001.

Yuan, J. C. and Shiller, A. M.: Determination of subnanomolar levels of hydrogen peroxide in seawater by reagent-injection chemiluminescence detection, Anal. Chem., 71(10), 1975–1980, doi:10.1021/ac981357c, 1999.

Zika, R. G., Moffett, J. W., Petasne, R. G., Cooper, W. J. and Saltzman, E. S.: Spatial and temporal variations of hydrogen peroxide in Gulf of Mexico waters, Geochim. Cosmochim. Acta, 49(5), 1173–1184, doi:10.1016/0016-7037(85)90008-0,
15  1985.

Zinser, E. R.: Cross-protection from hydrogen peroxide by helper microbes: The impacts on the cyanobacterium Prochlorococcus and other beneficiaries in marine communities, Environ. Microbiol. Rep., doi:10.1111/1758-2229.12625, 2018.