~~Trace chemical species in marine incubation experiments, part A.~~ Experiment design and bacterial abundance control extracellular H_2O_2 concentrations during 4 series of mesocosm experiments.

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Abstract

The extracellular concentration of H_2O_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_2O_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_2O_2 production and catalase/peroxidase enzyme production rates differ between species). Whilst there are extensive measurements of photochemical H_2O_2 formation rates and the distribution of H_2O_2 in the marine environment, it is poorly constrained how different microbial groups affect extracellular H_2O_2 concentrations, how comparable extracellular H_2O_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_2O_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 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 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.

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). Quantum yields for H_2O_2 formation increase with declining wavelength and so the ultraviolet (UV) portion of natural sunlight is a major source of H_2O_2 in surface aquatic environments (Cooper et al., 1988, 1994). Sunlight normalized H_2O_2 production rates therefore peak between wavelengths of 310-340 nm (Kieber et al., 2014). 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 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 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

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 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 2-10× higher than those found in the surface ocean (Morris and Zinser, 2013). We have previously hypothesized that the same may be generally true for meso-scale experiments (Hopwood et al., 2018b) 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; 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

Our rationale for the investigation of H_2O_2 trends during these 20-8000 L scale mesocosm and microcosm experiments is that the experiment matrixes for each experiment permitted the changing of 1,2 or 3 key variables (DOC, zooplankton, pH) whilst maintain others (e.g. salinity, temperature, light) in a constant state across the mesocosm/microcosm experiment. The relationships between H_2O_2 and other chemical/biological parameters are therefore potentially easier to investigate than in the ambient water column where mixing and the vertical/lateral trends in H_2O_2 concentrations must also be considered. Additionally, two of the experiment designs described herein (see Table 1) were repeated in 3 geographic locations facilitating direct comparisons between the experiment results with only limited mitigating factors concerning method changes.

~~1.1~~2.1 Mesocosm set up and sampling

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 (MesoPat, MesoArc and MesoMed~~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 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. For MesoPat and MesoArc~~In Svalbard, Patagonia and Gran Canaria~~ the mesocosms were tethered to a jetty. For MesoMed~~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 (without zooplankton, DOC, or nutrient additions) for calibration purposes and baseline measurements on day 0. During ~~the~~ MesoMed~~iterranean mesocosm~~, this surplus container was incubated alongside the mesocosms for the duration of the experiment without any further additions/manipulation.

Label (Project)	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
Multi~~eso~~Pat (Ocean Certain) ~~Multistressor~~	Comau fjord, Patagonia	Nov 2014	8	DOC, grazing, pH	20	Temperature controlled room	II	Final [H_2O_2]
Micro~~eso~~Pat (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
Multi~~eso~~Arc (Ocean Certain)	Kongsfjorden, Svalbard	July 2015	8	DOC, grazing, pH	20	Temperature controlled	II	Limited time series

~~Multistressor~~						room		
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
Multi~~eso~~Med (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

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Experiment	PAT (Patagonia)	ARC (Svalbard, Arctic)	MED (Crete, Mediteranean)	Gran Canaria
Mesocosm	MesoPat	MesoArc	MesoMed	Gran Canaria
Containers	HDPE 1000 L	HDPE 1250 L	HDPE 1500 L	Polyurethane 8000 L
Lighting	Ambient	Ambient	Ambient reduced ~50% with net	Ambient
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	MultiPat	MultiArc	MultiMed	
Containers	HDPE collapsible 20 L	HDPE collapsible 20 L	HDPE collapsible 20 L	
Lighting	36 W lamps	36 W lamps	36 W lamps	
Light regime	15 h light / 9 h dark	24 h light	15 h light / 9 h dark	
Zooplankton treatment	+30 copepods L^{-1}	+5 copepods L^{-1}	+4 copepods L^{-1}	
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	MicroPat			
Containers	HDPE collapsible 20 L			
Lighting	36 W lamps			
Light regime	15 h light / 9 h dark			
Containers Grazing treatment	HDPE collapsible 20 L +30 copepods L^{-1}			
Containers Grazing treatment	HDPE collapsible 20 L +30 copepods L^{-1}			

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.

The 10-mesocosm experiment design matrix was the same for ~~all 3 Ocean Certain (~~MesoPat, MesoArc and~~,~~ 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 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 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., 2018b). 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 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 (MicroPat) incubation mirroring the MesoPat 10 tank mesocosm (treatment design as per Fig. S1 I, but with 6 × 20 L containers per treatment -one for each time point- rather than a single HDPE tank) and three 16-treatment multistressor experiments (MultiPat, MultiArc and MultiMed 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

(MultiPat~~agonia~~, MultiArc~~Svalbard~~ and Multi Med~~iterranean~~) and the single microcosm incubation (MicroPat~~agonia~~), 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 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 MicroPat~~microcosm~~ incubation duplicated the MesoPat experiment~~mesocosm~~ design (Table 1B) and thereby consisted of 10 treatments. The experiment matrix for the 3 multistressor experiments (MultiPat, MultiArc and MultiMed outlined in Fig. S1 II) duplicated the corresponding mesocosm expeirments at the same fieldsites (MesoPat, MesoArc and MesoMed)~~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 (± 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~~ M~~m~~ultiMed~~stressor~~ 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 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., 2018b). For extensive datasets, the diurnal range of H_2O_2 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 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).

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 (MesoMed, MultiMed), 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 (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/MicroPat/MultiPat experiments was measured similarly as per Gran Canaria using m-cresol. During MesoArc/MultiArc/MesoMed/MultiMed 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/MultiMed), 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.

~~2.0~~3.0 Results

3.1 H_2O_2 time series during outdoor mesocosm incubations; MesoMed~~iterranean~~ 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 (ANOVA $p<0.05$ for all treatments compared to ambient conditions~~Fig. 1~~) . The mean and median ambient H_2O_2 concentration throughout the experiment was at least 40% lower than that in any mesocosm treatment (Fig. 1). This included the 400 µatm mesocosm which received no additions of any kind until the nutrient spike on day 18. 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.

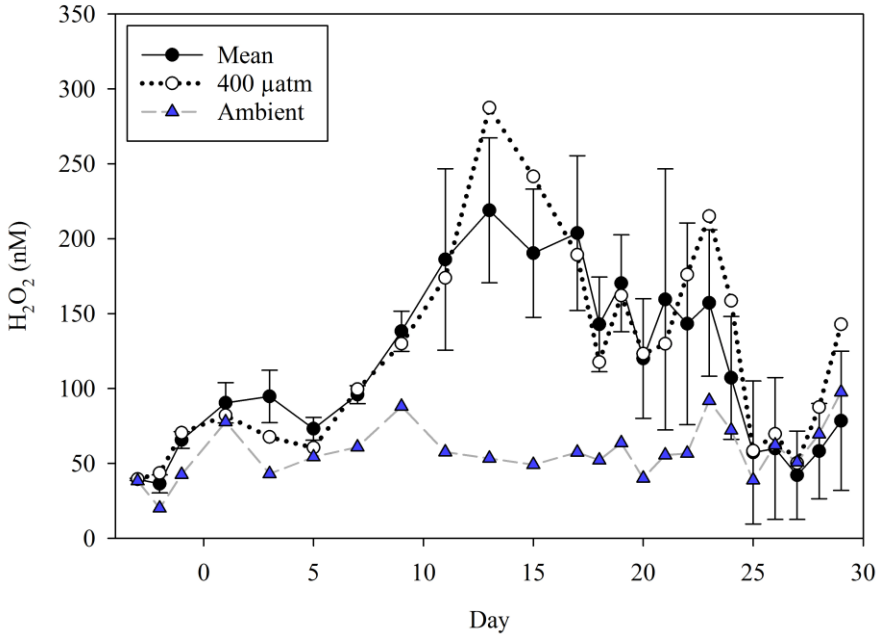

Figure 1: A summary of H_2O_2 over the duration of a pCO_2 gradient mesocosm in Gran Canaria. Data from Hopwood et al., (2018). The mean (± SD) ~~mesocosm~~ H_2O_2 from all pCO_2 treatments is contrasted with the concentration in ambient surface seawater

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)).

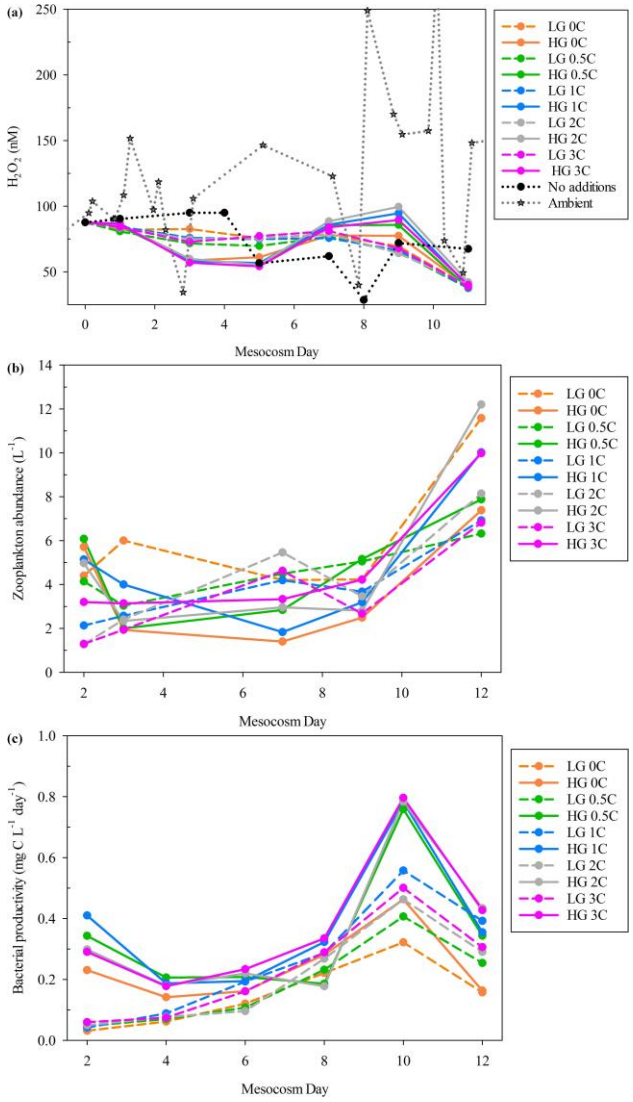

Figure 2: (a) H_2O_2 in all mesocosms during MesoMed in Gouves, Crete. A 10-treatment matrix (as per Fig. S1) was used (b) Zooplankton abundances showed a rapid convergence in the HG/LG status of the mesocosms after day 3 (c) The trend in bacterial productivity showed broad similarity within the HG and 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 MultiPat, MultiMed and MicroPat incubations ~~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.

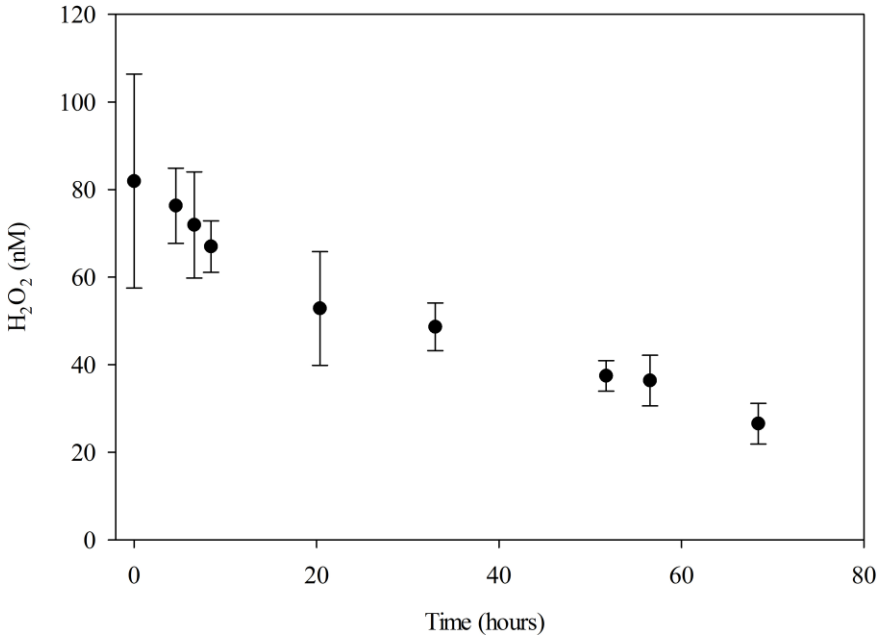

Figure 3: Seawater from ~~the~~ MesoMed ~~mesocosm~~ (without macronutrient, DOC or zooplankton amendment) was used to fill a 20 L HDPE container which was then incubated under the synthetic lighting used in the ~~Mediterranean multistressor~~ MultiMed experiment for 72 h with regular sub-sampling for analysis of H_2O_2.

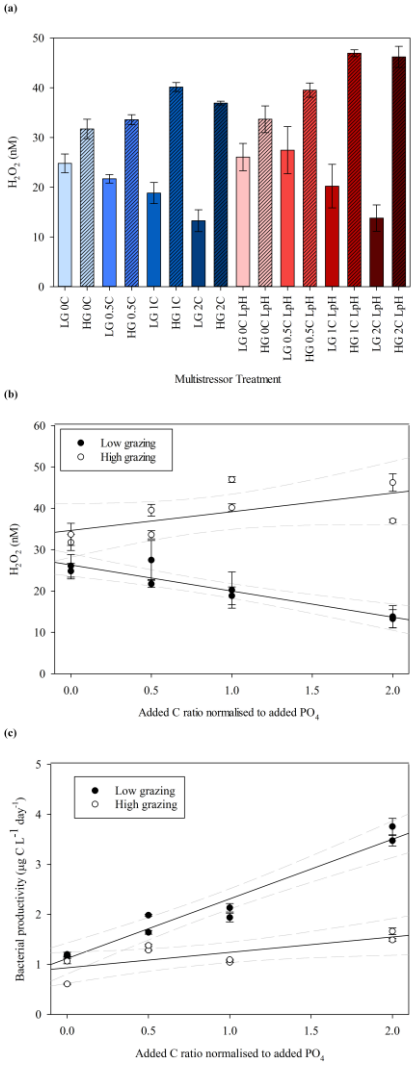

Figure 4: (a) ~~Multistressor~~ H_2O_2 concentrations at the end of the Multi~~Meso~~Med ~~multistressor~~ experiment (Day 9). Ambient pH (blue), low pH (red); high grazing (hashed); carbon (C) added at 0, 0.5, 1.0, 1.5 and 2.0 × Redfield carbon: phosphate ratio. (b) Plotting both ambient and low pH 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~~ ~~M~~multiMed~~stressor~~ 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 experiment~~mesocosm~~, a clear difference was noted between H_2O_2 concentrations in the high and low zooplankton addition treatments (Fig. 4 (b)), with the high grazing always resulting in higher H_2O_2 concentrations ~~(Fig. 4 (b))~~ (t test, $p < 0.001$). 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 operated in the opposite direction (Fig. 4 (b)). In high grazing treatments, increasing C corresponded to increasing extracellular H_2O_2 concentrations (linear regression coefficient 4.5 ± 2.3); whereas in low grazing treatments, increasing C corresponded to decreasing extracellular H_2O_2 concentrations (linear regression coefficient -6.3 ± 0.97). Bacterial productivity increased with added C in both high (linear regression coefficient 0.31 ± 0.1) and low grazing treatments (linear regression coefficient 1.2 ± 0.1), but there was a more pronounced increase under low grazing conditions (Fig. 4 (c)).

At the end of the ~~Patagonia~~ ~~M~~multiPat experiment~~stressor~~ (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). However, two of the low pH treatments were outliers (defined as 1.5 IQR) with particularly high H_2O_2. Without these two datapoints, there would be no significant difference between in high and low treatments (p=0.39). Contrary to the results from the ~~same experiment in the~~ MultiMed experiment~~iterranean~~ (Fig. 4), there was no significant difference between high/low grazing treatments (Mann-Whitney Rank Sum test p ~~=~~ =0.65). 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).

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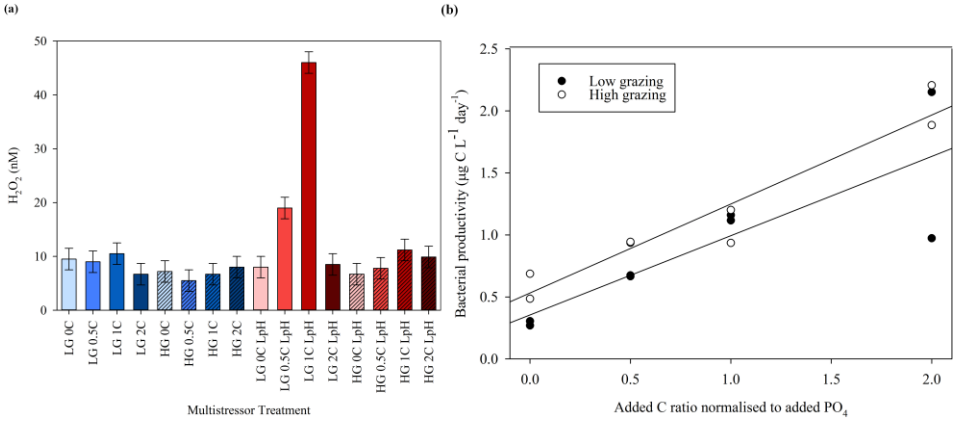

Figure 5: (a) ~~Multistressor~~ H_2O_2 concentrations at the end of the M~~ulti~~es~~o~~Pat ~~multistressor~~ experiment. Normal pH (blue), low pH (red); high grazing (hashed); DOC added at 0, 0.5, 1.0, and 2.0 × Redfield carbon (C):phosphate ratio indicated by increasing colour density. (b) Plotting 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~~ M~~n~~icroPat~~cosm~~ 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 MultiPat~~agonia~~ (Fig. 5 (b)) or MultiMed~~iterranean~~ (Fig. 4 (c))~~ multistressor~~ experiments.

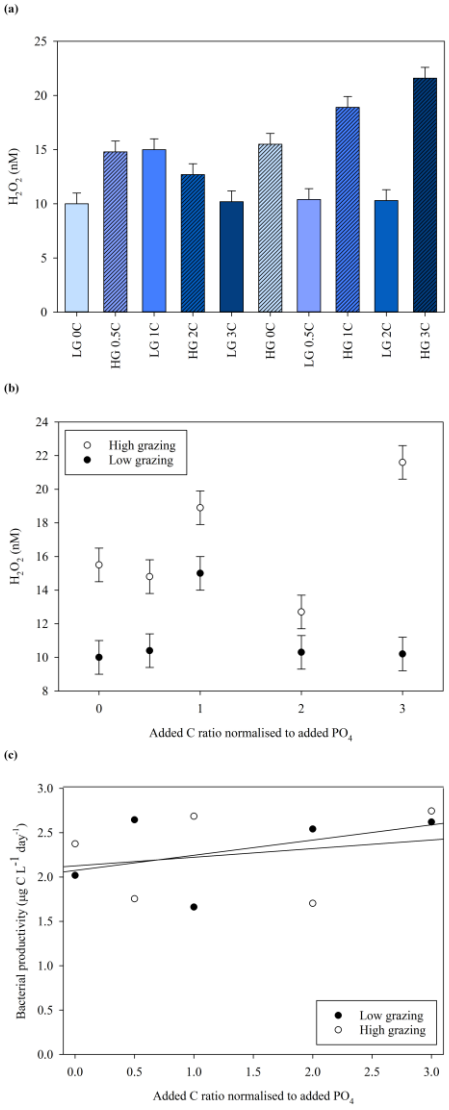

Figure 6: (a) ~~Microcosm~~ H_2O_2 concentrations at the end of the Micro~~Meso~~Pat ~~microcosm~~ experiment. High grazing treatments are hashed; DOC added at 0, 0.5, 1.0, 2.0 and 3.0 × Redfield carbon (C):phosphate ratio indicated by increasing colour density. (b) No clear trend was evident across 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., 2018b). 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.

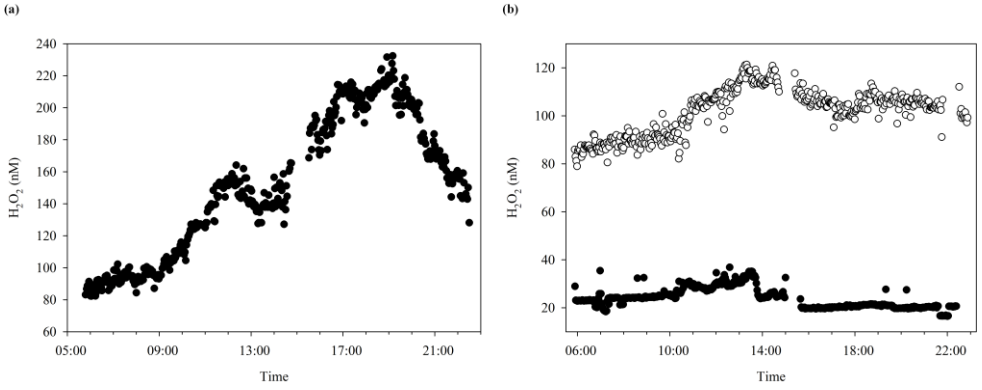

Figure 7: (a) Diurnal cycling of H_2O_2 in coastal seawater (Gouves, Crete 17 May) and (b) in the no addition tank (number 11) during the MesoMed mesocosm on May 15 (open circles) and May 19 (closed circles) 2016 (experiment days 4 and 8, respectively).

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 MesoPat/Arc/Med, MicroPat and MultiPat/Arc/Med~~Ocean Certain~~ experiments all 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 ~~(~~Hopwood, 2018~~)~~ ~~'Part B'~~) also appeared to be unaffected by the copepod density as the difference between treatments was almost negligible (<0.04 nM).

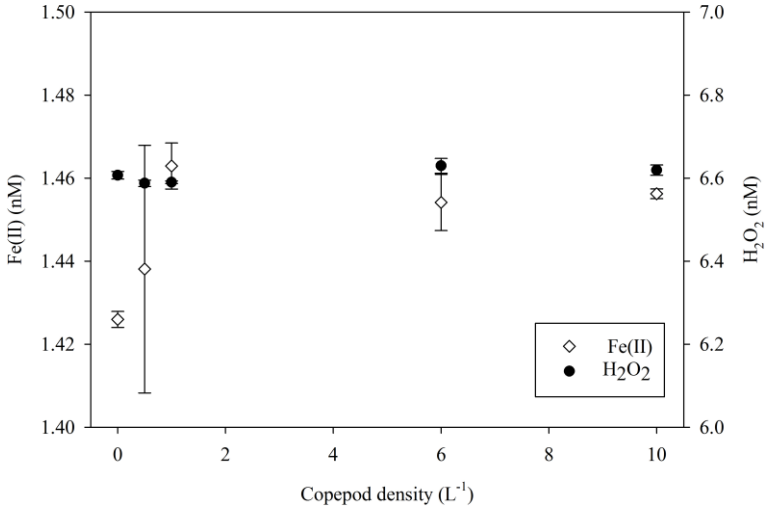

Figure 8: H_2O_2 and Fe(II) concentrations in a culture of diatoms growing in coastal seawater after 20 h of incubation with a zooplankton gradient imposed by addition of copepods.

At the end of the Meso~~iterranean mesocosm~~ experiment, seawater (extracted from the baseline treatment from the mesocosm on day 11) was used in two side experiments. During both the extracellular H_2O_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_2O_2 concentration gradient used in each experiment was determined by considering the ambient concentration of H_2O_2 in the mesocosms (e.g. Fig. 2) and in ambient seawater close to the mesocosm facility. After the first daily H_2O_2 measurements were made, the required spikes to maintain the desired H_2O_2 gradient were calculated based on measured rates of H_2O_2 decay. H_2O_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 Multi~~iterranean microcosm~~ experiment. H_2O_2 concentrations again verified that manipulation with H_2O_2 spikes

successfully created a high, medium and low H_2O_2 treatment (mean for triplicate low/medium/high treatments: 40 ± 2, 120 ± 6, 230 ± 7 nM H_2O_2). Bacterial production showed no statistically significant (ANOVA, p~~P~~ = ~~–~~0.562) difference between triplicate low (1.69 ± 0.28 µg C L^{-1} day^{-1}), medium (1.30 ± 0.60 µg C L^{-1} day^{-1}) and high (1.29 ± 0.56 µg C L^{-1} day^{-1}) H_2O_2 treatments.

For a concurrent manipulation in the Mediterranean using 20 L HDPE containers incubated outdoors, a gradient in H_2O_2 concentrations was similarly imposed. These manipulations successfully produced a clear gradient of H_2O_2 conditions with relatively consistent H_2O_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 × 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)).

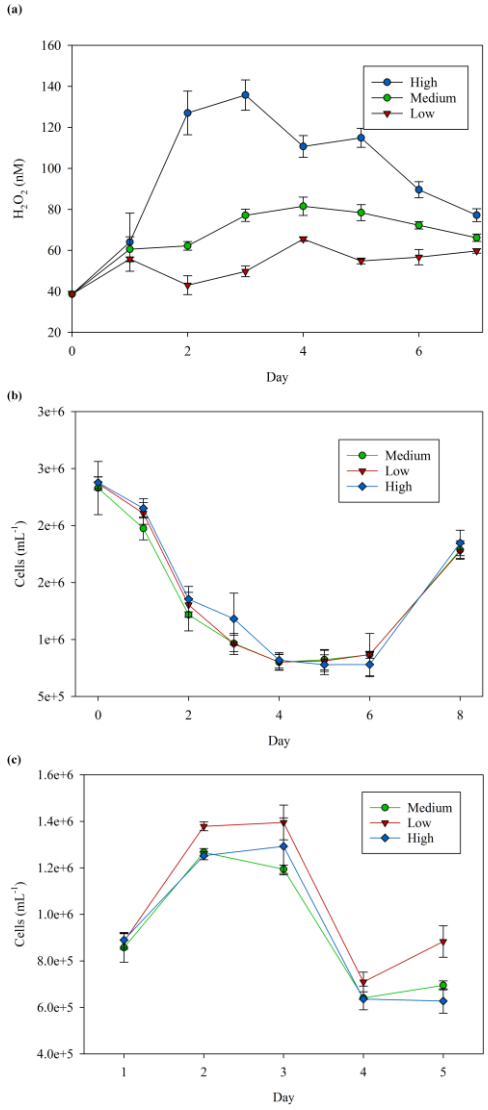

Figure 9: (a) H_2O_2 gradient during the 20 L scale Mediterranean side experiment where a H_2O_2 gradient was created with H_2O_2 spikes and catalase (b) bacteria abundance during the same Mediterranean experiment (c) bacteria abundance for a similar incubation in Gran Canaria. Mean and standard deviations of triplicate treatments are plotted in all cases.

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 ± 1 nM (mean of all 3 × 3 replicates at day 0), confirmed that a gradient was maintained over the 5-day duration of the experiment (mean 210 ± 113, 62 ± 14 and 47 ± 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 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

4.1 Bacteria, zooplankton and extracellular H_2O_2 trends

During all multi/micro/meso 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).

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 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., 2018b). In the incubation experiments, whenever there was a sustained difference in extracellular H_2O_2 concentrations between treatment groups (MesoMed Fig. 2 and MultiMediterranean 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 MmultiPatstressor (Fig. 5) and MmicroPatcosm (Fig. 6) incubations showed 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 herein (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~~ MultiPat~~agonian 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 M~~~~m~~ultiPat~~stressor~~ was not particularly low. A more distinct difference however arose in bacterial productivity (Fig. 5 (b)). Unlike MesoMed, the MultiPat~~agonian multistressor~~ and M~~m~~icroPat~~cosm 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 (MesoPat, MicroPat and MultiPat) 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., 2018b). During MesoMed, increases in zooplankton abundance coincided with decreases in H_2O_2 concentration (Fig. 2). Similarly, during MultiMed~~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.

Bacteria are expected to be a dominant H_2O_2 sink in most aquatic environments (Cooper et al., 1994). Here 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). A key reason for this may simply be the generally low H_2O_2 concentrations measured in most of our experiments. At the low

H_2O_2 concentrations of <50 nM observed during most experiments, the influence of any parameter on H_2O_2 removal would be more challenging to determine from an analytical perspective due to reduced signal:noise ratio. However, the H_2O_2-defence mechanism of organisms may also be sensitive to ambient H_2O_2 concentrations. (Morris et al., (2016) suggest that microbial communities exposed to high H_2O_2 have elevated H_2O_2 defences. If the microbial communities here exhibited a dynamic response to H_2O_2 concentrations in terms of their extracellular H_2O_2 removal rates, this would dampen the correlation between bacterial abundance and H_2O_2 concentrations- especially at low H_2O_2 concentrations. Combing all available H_2O_2 concentrations for which the corresponding total bacterial cell counts areabundance is available (Fig. 10) from all experiments (except the side experiments where H_2O_2 was manipulated using catalase or H_2O_2 spikes), provides some limited evidence for the dominance of bacteria as a H_2O_2 sink. ~~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),~~ tThere was a notable absence of high-H_2O_2, high-bacteria datapoints in any experiment (Fig. 10). The observed distribution is therefore consistent with a scenario where bacteria dominate H_2O_2 removal, but other factors (possibly including experiment design, see s4.2) can also lead to low H_2O_2 conditions independently of bacterial abundance.

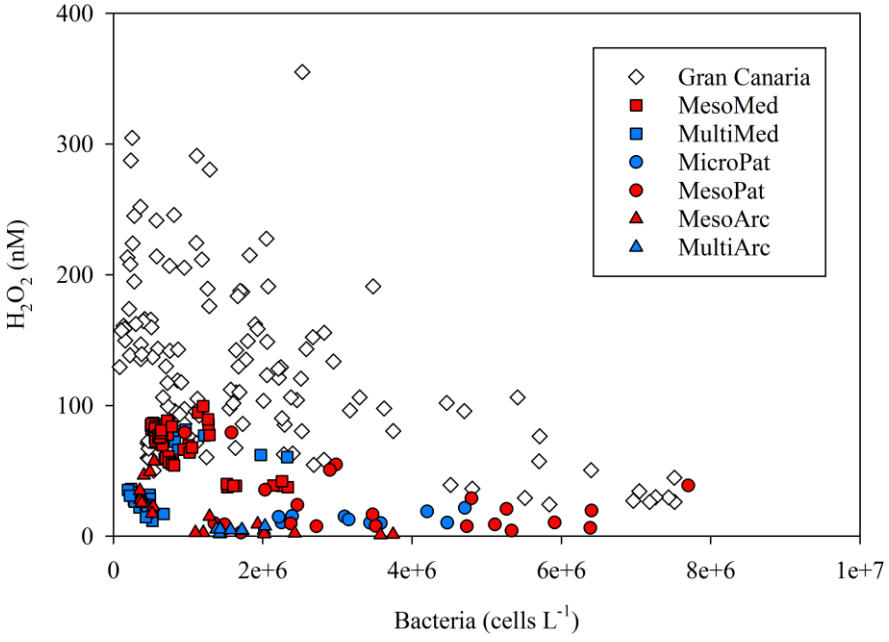

Figure 10: Bacterial cell counts and H_2O_2 for all available data from all incubation experiment time-points where both measurements were made within 24 h of each other.

4.2 Changes in extracellular H_2O_2 due to experiment design

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 (MultiPat/Arc/Med and MicroPat) 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)). This is not surprising considering the light arrangements for these experiments (Table 1). The Gran Canaria experiment was practically unshaded with surface seawater exposed to natural sunlight. The closed HDPE mesocosms (MesoMed, MesoPat, MesoArc) experienced natural sunlight but after attenuation through 1-2 cm of HDPE plastic. Whilst the transmission of different light wavelengths through these HDPE containers was not tested during our experiments, 1-2 cm of polyethylene should strongly attenuate the UV component of sunlight. The 20 L scale experiments (MultiMed, MultiPat, MultiArc and MicroPat) were conducted using identical synthetic lighting with lamps selected to as closely as possible replicate the wavelength distribution of natural sunlight. However, the fluorescent light distribution is still deficient, relative to sunlight, in wavelengths <400 nm, which is the main fraction of light that drives H_2O_2 formation in surface seawater (Kieber et al., 2014), and these containers still mitigated the limited UV exposure with a 1 mm HDPE layer which would further reduce the UV component of incoming light.

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, Svalbard	July 2015	78.9° N	9.0-35.2	5.0-9.0	10-100

Table 2. Range of water properties in freshly collected coastal seawater ~~adjacent to mesocosms~~. 'NA' not applicable. [a] Temperature of pool facility at HCMR, [b] Coastal seawater approximately 500 m from HCMR facility.

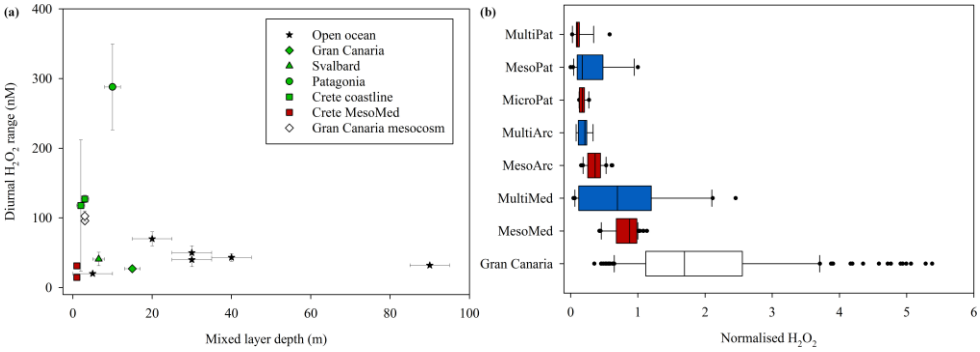

Figure 11: (a) Observed diurnal ranges in H_2O_2 concentrations. Black stars show literature surface marine values and green shapes in-situ experiments corresponding to experiment field site locations (b) H_2O_2 across all experiments as a fraction of ambient H_2O_2. For the Meso/Multi ~~Ocean Certain~~ fieldsites (Mediterranean, Arctic and Patagonia) red bars~~shapes~~ are outdoor mesocosms and blue shapes indoor incubations. Median, $10^{th}/25^{th}/75^{th}/90^{th}$ 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 further offshore surface waters of the Atlantic, Gulf of Mexico and sub-tropical equatorial Pacific along the Peruvian shelf 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)).

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. MesoMed~~diterranean~~), yet enhanced in open polyurethane bags (Gran Canaria). During the ~~Ocean Certain~~ multistressor and microcosm experiments, incubated indoors in 20 L HDPE containers,

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

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 a side experiment after MesoMed~~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 for the MesoMed side experiment~~in Crete the experiment was run without a~~ no macronutrient spike was added), 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 and at nanomolar concentrations (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 when more subtle changes are made to ambient H_2O_2 concentrations. 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 oxidative stress associated with high H_2O_2 concentrations- which is a communal benefit (Zinser, 2018). Yet~~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 directly than autotrophs from the enhanced stability of labile DOM under low H_2O_2 conditions. However, whilst H_2O_2 is a reactive species, at the concentrations present in the marine environment the direct effects of changing H_2O_2 concentration on the abundances of different microbial groups (e.g. Fig. 9) are clearly minor. A specific challenge with determining the effect(s) of H_2O_2 concentration on any biogeochemical processes, and vice-versa, is that the diurnal variability in H_2O_2 concentration is always large compared to inter-treatment differences in H_2O_2 concentration within individual experiments (e.g. Fig. 11). High resolution data is therefore clearly required to properly interpret H_2O_2-microbial interactions and to better quantify the subtle links between H_2O_2 cycling and microbial functioning.

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 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 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 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 with input from other authors.

5 Acknowledgements

The Ocean Certain and KOSMOS/PLOCAN teams assisting with all aspects of experiment logistics and organisation are thanked sincerely for their efforts. Labview software for operating the H_2O_2 FIA system was designed by P Croot, M Heller, C Neill and W King. Financial aid from the European Commission (OCEAN-CERTAIN, FP7- ENV-2013-6.1-1; no: 603773) is gratefully acknowledged. JA was supported by a Helmholtz International Fellow Award, 2015 (Helmholtz Association, Germany).

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Note that in addition to the comments by reviewers on this text, a companion manuscript concerning a different aspect of the same mesocosm experiments was also recently reviewed for this journal. As it is highly desirable to have a consistent use of terminology between these (and other in prep.) texts concerning the experiment set up, the following change has also been made to this text in order to maintain consistency: The names of the major experiments has been standardized throughout the text and we have been careful to use only one specific term of reference for each experiment. The mesocosm/microcosm/mutlistressor experiments are now termed MesoPat/MesoArc/MesoMed/MultiPat/MultiArc/MultiMed/MicroPat/Gran Canaria.

(Previously the term 'MesoPat' was used to refer to the field campaign which included a trio of mesocosm/multistressor/microcosm experiments, but this was found to be confusing, 'MesoPat' now refers exclusively to the 1000 L scale mesocosm experiment conducted in Patagonia).

Anonymous Referee #1 Received and published: 24 July 2018
The goal of this study was to determine if aspects of an experimental design could inadvertently affect the photochemical or biological production of hydrogen peroxide (H2O2), thus altering the outcome of the study. This was tested by analyzing the compiled data from multiple coastal mesocosm experiments and determining which factors or aspects of the experimental design caused a change in H2O2 concentration compared to the ambient concentration found in surrounding seawater. Based upon their analysis, the authors concluded that the isolation of seawater within a mesocosm, alterations to light intensity, and changes to bacterial abundance were responsible forvariations in H2O2 concentration between the mesocosm vessels and the surrounding seawater. This study represents an interesting opportunity to observe how standard methods of experimental design (mesocosms) could potentially influence experimental outcomes in marine environments. Additionally, this study is unique in how the authors explore the effect of organisms of higher trophic levels upon H2O2 concentrations. The authors were able to provide convincing evidence supporting the importance of bacterial communities in modulating H2O2 concentrations in the ocean.

Major comments: A major conclusion of the paper is that light treatment (ambient versus artificial) has a big impact on the H2O2 concentrations in the mesocosm experiment. While this is supported by the figures, it is difficult to tell which light treatments are used for each figure, and there is no indication in Table 1 if the mesocosms are exposed to sunlight or light bulbs.

Reply: We have made this important clarification throughout the text. Extra lines are added in Table 1 to state the exact light 'setup' for each experiment and within the text we have clarified which experiments were outdoor/indoor lighting arrangements.

Along these lines, there is essentially no discussion of the differences in light exposure, particularly the ability of UV in sunlight to generate the H2O2, and this should be mentioned in both the introduction and the discussion.

Reply: Information is added to the introduction to briefly outline the concept, "Quantum yields for H_2O_2 formation increase with declining wavelength and so the ultraviolet (UV) portion of natural sunlight is a major source of H_2O_2 in surface aquatic environments (Cooper et al., 1988, 1994). Sunlight normalized H_2O_2 production rates therefore peak between wavelengths of 310-340 nm (Kieber et al., 2014)." **Additionally, we further add a description of the lighting different and the ability of HDPE to remove/reduce UV light in the discussion,** "….considering the light arrangements for these experiments (Table 1). The Gran Canaria experiment was practically unshaded with surface seawater exposed to natural sunlight. The closed HDPE mesocosms (MesoMed, MesoPat, MesoArc) experienced natural sunlight but after attenuation through 1-2 cm of HDPE plastic. Whilst the transmission of different light wavelengths through these HDPE containers was not tested during our experiments, 1-2 cm of polyethylene should strongly attenuate the UV component of sunlight. The 20 L scale experiments (MultiMed, MultiPat, MultiArc and MicroPat) were conducted using identical synthetic lighting with lamps selected to as closely as possible replicate the wavelength distribution of natural sunlight. However, the fluorescent light distribution is still deficient, relative to sunlight, in wavelengths <400 nm, which is the main fraction of light that drives H_2O_2 formation in surface seawater (Kieber et al., 2014), and these containers still mitigated the limited UV exposure with a 1 mm HDPE layer which would further reduce the UV component of incoming light…"

The authors attempt to demonstrate how aspects of an experimental design (structure of vessel, setup, nutrient addition, increased stress) could affect the concentration of H2O2. While changes in H2O2 are measurable in all mesocosm experiments and are potentially attributable to a particular aspect of the experiment, the observed changes in H2O2 concentration are small with respect to total daily production of H2O2. All but one of the mesocosm experiments have H2O2 concentrations below 100nM and ranges of variation between 20-50nM. The prospect of changes in H2O2 concentration such as these recorded altering experimental outcome for microbial activity and DOC decay seems unlikely, without cited support.

Reply: These changes are certainly small and it is doubtful that the variation between different treatments within the mesocosms/multistressor experiments had measurable effects. However the side experiment in Gran Canaria did suggest a positive effect on bacteria when water was subject to a H2O2 decline equivalent to the 'gap' between natural and incubated water during some of these experiments. Nevertheless, we acknowledge that diurnal changes in H2O2 are large, and this large variation complicates any data interpretation about temporal changes in daily mean H2O2. This is now explicitly stated in the text, "A specific challenge with determining the effect(s) of H_2O_2 concentration on any biogeochemical processes, and vice-versa, is that the diurnal variability in H_2O_2 concentration is always large compared to inter-treatment differences in H_2O_2 concentration within individual experiments (e.g. Fig. 11)….."

Pg. 18 lines 24-26 – As stated here, no clear trends can be defined between H2O2 concentration and grazer abundance when considering all datasets used. Perhaps it would be beneficial to focus more intently upon the aspect of bacterial abundance and its effect upon H2O2 concentrations instead? Along with above comment, bacterial abundance is an integral part of

this study's conclusions yet only 2 figures give any data on how their abundances are changing. Inclusion of cells count data for the other experiments and datasets would strengthen this major argument of the paper.

Reply: This is perhaps clear after we present the data. The logic behind a focus on zooplankton/pH/DOC was that these were gradients which were present in all experiments that could [we thought] plausibly affect equilibrium H2O2 concentrations. It wasn't clear until after looking at the data that no clear effect of zooplankton (or pH) on H2O2 was evident. We presently show bacterial productivity data for all experiments and are not sure that it is necessary to plot cell counts and productivity separately in addition to the synthesis of all data (Fig. 10). In the case of bacteria as a H2O2 sink, an additional complication is the very low H2O2 concentrations at the end of all MultiPat/Arc/Med experiments which makes it challenging to find changes in [H2O2] due to the reduced signal:noise ratio. More importantly, there is also a biological issue here (which we now mention in the text – our discussion concerning the role of bacteria (s 4.1) is expanded), because microbial organisms may adapt the strength of their oxidative defenses to ambient H2O2 concentrations i.e. cellular H2O2 defences are less active at lower H2O2 concentrations. Even for those experiments were detailed counts (total, or species level), are available, it therefore becomes difficult to make any valid argument concerning cell counts and group/species level abundances at these low H2O2 concentrations as the relationship between the two would only likely be observed at higher H2O2 concentrations. "the H_2O_2-defence mechanism of organisms may also be sensitive to ambient H_2O_2 concentrations. Morris et al., (2016) suggest that microbial communities exposed to high H_2O_2 have elevated H_2O_2 defences. If the microbial communities here exhibited a dynamic response to H_2O_2 concentrations in terms of their extracellular H_2O_2 removal rates, this would dampen the correlation between bacterial abundance and H_2O_2 concentrations- especially at low H_2O_2 concentrations…."

Minor comments:
The authors claim that the isolation of seawater in mesocosm vessels allows for the accumulation of H2O2. This is discussed throughout the manuscript but notably in Figure 1. on pg. 9 line 22-32 and pg. 21 line 1-11. In Figure 1, the authors claim that there is no clear trend between H2O2 and pCO2 concentration, leading them to conclude that changes in H2O2 are due to the enclosure used to house the water. Does this graph show H2O2 concentrations in unamended seawater within one of the polyurethane bags used, i.e. is the baseline 400atm a control? If not, then H2O2 production cannot solely be attributed to the container used. In Figure 1 is it possible that the microbes are nutrient depleted by day 8-9, and the increase in H2O2 is due to their decline in abundance? This would also explain why the H2O2 concentration decreases around day 18 when the nutrient addition was made.

Reply: for the experiment shown in Figure 1, yes the 400 atm 'treatment' is a control in this sense i.e. atmospheric PCO2 with no additions of CO2 made (and no other additions of any kind before the nutrient spike on day 18).

Axis labels throughout manuscript are misleading. H2O2 / nM should be shown as H2O2 (nM), etc. In Figure 2 panel a, the H2O2 concentrations for ambient seawater and LG 2C treatment are difficult to discern. Consider a different representation of the data.

Reply: amended accordingly.

Pg. 20 lines 15-20
– The authors are comparing H2O2 production ranges from open ocean environments to those measured in coastal environments.

Reply: This is now explicitly stated in the text, but does not really affect our interpretation. The key point was that some diurnal ranges in mesocosms are very high (higher than expected based on diurnal ranges in the same location) whereas some diurnal ranges in mesocosms are very low (based on diurnal ranges in the same location). The offshore values are shown for comparison only to help interpret the data.

In Table 2 on pg. 20, the upper H2O2 concentrations listed for the Crete and Patagonia locations are significantly higher than any data shown in previous figures from those same locations.

Reply: These refer to 'natural' seawater outside the experiments and are included for reference only to compare to the experimental results. This further clarified both in the text and in the abstract to avoid confusion.

Pg. 21 lines 13-14 – Were individual microbial groups ever quantified? Or was this observation made from total cell counts?

Reply: For these experiments groups were quantified.

Figures 4a and 5a: are these data from the same experiment? The values for "LG 1C" look different in these figures, as one example.

Reply: No they are different datasets. 4(a) is MultiMed. 5(a) is MultiPat. We have reformatted the figure descriptions to highlight the experiment names better and avoid confusion.

Ma (Referee) Received and published: 30 November 2018
This work provides large scale mesocosm experiments to elucidate how microbial groups affect extracellular H2O2 concentrations and other related questions. It has shown that the high bacterial densities were associated with low H2O2. This manuscript generally reads well and presents a good rationale of research. However, the study could be significantly improved with the addition of missing details on the methodology used in experiment design, as well as statistical support. The major issue is that there are so many variables in this work, which have not been fully considered regarding to the result interpretation. All these variables could play a great role in affecting the extracellular H2O2 concentration while the rationale to use these variables were not explained well and when the conclusion could not be obtained between microbial groups and H2O2 concentrations if all other variables were playing great role in it. These variables include (not limited to): zooplankton concentrations, different bacterial community, temperature, nutrient (concentrations and chemicals), light (light cycle and light intensity), DOC and pH. For example: In Glippa et al., 2018, "Vehmaa et al. [21] found that a 3 degrees rise in temperature increased the antioxidant capacity (ORAC, Oxygen Reactive Absorbance Capacity) in Acartia copepods by almost 15%, and they measured a 2-fold increase also in oxidative damage, measured as lipid peroxidation".

Reply: There are of course many variables which exert influence on extracellular H2O2 concentrations. One the main rationale for working with mesocosm experiments was that intra-experiment data is free from variation in some of these variables. Salinity/temperature/light exposure/nutrient addition are close to constant across the mesocosm units within each experiment. We have added a paragraph to explain this rationale (below). Concerning between-experiment differences, these are of course more challenging to explain because there are differences in physical/biogeochemical parameters between fieldsites. This is a main reason why we attempted to 'normalize' data to ambient H2O2 concentrations as this (and some tests on our experiment setup) provides the strongest evidence that low H2O2 across many of the experiments arises simply from the plastic containers used rather than 'natural' parameters. "….our rationale for the investigation of H_2O_2 trends during these 20-8000 L scale mesocosm and microcosm experiments is that the experiment matrixes for each experiment permitted the changing of 1,2 or 3 key variables (DOC, zooplankton, pH) whilst maintain others (e.g. salinity, temperature, light) in a constant state across the mesocosm/microcosm experiment. The relationships between H_2O_2 and other chemical/biological parameters are therefore potentially easier to investigate than in the ambient water column where mixing and the vertical/lateral trends in H_2O_2 concentrations must also be considered. Additionally, two of the experiment designs described herein (see Table 1) were repeated in 3 geographic locations facilitating direct comparisons between the experiment results with only limited mitigating factors concerning method changes."

Specific comments:
The line numbers started over on each page. It is better to have continuous line number from the beginning to the end of the manuscript.

Reply: Changed in Revised text.

P9 L27: Is there statistics to support the "H2O2 was generally elevated"?

Reply: A line is now added in the revised text. In this particular case, the difference was so large we didn't think it necessary to detail ANOVA results, the mean/median ambient level is at least 40% lower than any treatment.

P11 L9-L10: It is hard to get the conclusion of "this trend closely matched that observed in zooplankton biomass" by only eyeballing it, especially when the 5th day of zooplankton biomass was not shown in the figure.

Reply: a reason why there is no statistical test here is because, for logistical reasons which we acknowledge are not ideal, the zooplankton biomass data and the H2O2 data are at different timepoints. There isn't a 'missing' datapoint, there is simply a lower resolution for zooplankton data in this experiment and a temporal mismatch between the two data series. One of the experimental problems, which we raise in the text already is that any inter-day temporal trend in [H2O2] made using 'spot' measurements must be done at the same time daily. Where possible (and basically wherever there are stats present in the manuscript), we timed the measurement of all parameters to be the same so that we can directly compare [H2O2] to other parameters and report [H2O2] at the same time daily. However, for some parameters, including zooplankton during MesoMed, such a coherent timing simply wasn't possible due to the significant amount of time required to sample these parameters from the mesocosms. In these experiments, where we can only comment on the general trend, we have rephrased the text to highlight the uncertainty. The line referred to (P11 L9-10) is removed.

P12 L13: Statistics would be helpful to support "a clear difference was noted between".

Reply: t test added comparing the two groups (p <0.001) accordingly.

P13 L7-L8: Again statistics would be needed to the statement "there was a more pronounced increase".

Reply: regression details added (HG 0.31 ± 0.1, LG 1.2 ± 0.1) accordingly.

P13 L1-L13: Regarding to the statements, "In the low pH treatment (initially 7.54 _ 0.09), H2O2 concentrations were significantly higher (Mann-Whitney Rank Sum test p 0.02) compared to the unmodified pH treatment (initially 8.01 _ 0.02)". Only by eye-balling it, it showed the LG0.5C LpH and LG 1C LpH have higher concentration of H2O2. Is this statement based on only these two data points? Regarding to the statistics p value, it would be helpful if it is equal to, less than or greater than some certain number by indicating with corresponding symbols.

Reply: P values are now labelled < / > / = . Yes there are two very high H2O2 values in this dataset, both of which happen to be low pH/medium carbon treatments. If these values are excluded then the significance of the difference between low pH and high pH treatments disappears. Whilst there are only a limited number of datapoints in each (low/high) pH category, these two can be defined as anomalies based on 1.5 IQR if we look at the low pH and normal pH sets as groups of 8. This is now noted in the text.

P15 L8-L13: It would be great to put these discussions after (Table 1) under Discussion.

Reply: amended.

P16 L16-L17: Regarding to this statement, "Bacterial production showed no statistically significant (ANOVA, P 0.562) difference between low, medium and high H2O2 treatments.", there is no data to support it. Is it related with Fig. 9(c)?

Reply: No this is a separate side experiment. We had included a figure to show these data but dropped it to save space. The values (triplicate ± SD) are now provided within the text…
"Bacterial production showed no statistically significant (ANOVA, p=0.562) difference between triplicate low (1.69 ± 0.28 µg C L^{-1} day^{-1}), medium (1.30 ± 0.60 µg C L^{-1} day^{-1}) and high (1.29 ± 0.56 µg C L^{-1} day^{-1}) H_2O_2 treatments"

P17 L3: The author claimed there is NO significant difference while the p value is less than 0.05.

Reply: Typo corrected, should have been '> 0.05' not '< 0.05'

Figure 1: There is line to indicate the Mean H2O2. However, it is not clear on how to get this Mean.

Reply: Clarified in the figure label…. "Data from Hopwood et al., (2018). The mean (± SD) H_2O_2 from all 8 pCO_2 treatments is shown"

Figure 2: Is there any interpretation on the big variation of H2O2 in ambient? Is there replicates to have error bar? Statistics would be helpful here to show the difference between HG/LG status.

Reply: We can of course speculate. The 'ambient' measurements always refer to the coastal ocean. Unlike the other fieldsites (Svalbard, Patagonia, Gran Canaria), this location (for the Mediterranean/Crete experiments) was not a sheltered fjord or harbor which likely means the H2O2 is much more variable due to changing stratification in the water column. But as we only

sampled surface water at intervals during the experiment we can't really quantify this or do anything other than speculate about the underlying causes.

The discussion of the zooplankton trend is now not explicitly linked to H2O2 (see comment above). Noting the different timing of the measurements during this specific experiment it is not possible to produce meaningful statistics.

There are replicate measurements for all ambient water measurements, which produce a very small error bar (1-5%). However, given the short-term changes to H2O2 that can occur in a dynamic water column even on very short (minutes) timescales (as demonstrated in our high resolution diurnal time series) we thought that plotting error bars based on analytical error for spot measurements would be misleading as it is not inclusive of the changes to [H2O2] that occur in natural waters over a time period equivalent to the sample collection/measurement time of 10-20 minutes.

Figure 7: It would be great to show diurnal cycling of H2O2 in two continuous days.

Reply: It would, but when the apparatus is set up to produce continuous data like this an analyst has to check on the instruments very regularly. It simply wasn't possible here to have them operating for more than 24 hours! We may try a different instrument/sensor configuration to achieve this in the future with slightly lower resolution and an auto-clean cycle.