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ORIGINAL ARTICLE

Comparative Response of Marine Microalgae to H_2O_2-Induced Oxidative Stress

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Abstract

There have been growing interests in the biorefining of bioactive compounds from marine microalgae, including pigments, omega-3 fatty acids or antioxidants for use in the nutraceutical and cosmetic sectors. This study focused on the comparative responses of five marine microalgal species from different lineages, including the dinoflagellate *Amphidinium carterae*, chlorophyte *Brachiomonas submarina*, diatom *Stauroneis* sp., haptophyte *Diacronema* sp. and rhodophyte *Rhodella violacea*, to exposure during their batch growth to hydrogen peroxide (H_2O_2). *A. carterae* returned an enhanced signal with the DPPH assay (8.8 µmol Trolox eq/g DW) when exposed to H_2O_2, which was associated with reduced pigment yields and increased proportions in saturated C16 and C18 fatty acids. *B. submarina* showed enhanced antioxidant response upon exposure to H_2O_2 with the DPPH assay (10 µmol Trolox eq/g DW), a threefold decrease in lutein (from 2.3 to 0.8 mg/g) but a twofold increase in chlorophyll b (up to 30.0 mg/g). *Stauroneis* sp. showed a downward response for the antioxidant assays, but its pigment yields did not vary significantly from the control. *Diacronema* sp. showed reduced antioxidant response and fucoxanthin content (from 4.0 to 0.2 mg/g) when exposed to 0.5 mM H_2O_2. *R. violacea* exposed to H_2O_2 returned enhanced antioxidant activity and proportions of EPA but was not significantly impacted in terms of pigment content. Results indicate that H_2O_2 can be used to induce stress and initiate metabolic changes in microalgae. The responses were however species-specific, which would require further dosage optimisation to modulate the yields of specific metabolites in individual species.

Keywords Microalgae · Hydrogen peroxide · Antioxidants · Pigments · FAMEs

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Research Highlights

- Comparative response to H_2O_2 treatment assessed for 5 microalgal species from different lineages.
- Results showed an enhanced antioxidant response for *Rhodella violacea* and an overall reduction for the other species.
- Pigment yield reduction was observed for most species except for chlorophyll b, which was enhanced in *Brachiomonas submarina*.
- Species-specific variations in the proportions of fatty acids were observed.
- Principal component analysis clearly separated the H_2O_2-treated *Rhodella violacea* set from the others, with higher antioxidant response and proportions of EPA.

Introduction

Marine microalgae have fostered increasing interests as promising feedstocks for producing bioenergy, bio-fertilisers, nutraceuticals or pharmaceuticals [1]. These microorganisms have received considerable attention due to their perceived advantages over higher plants in terms of not competing for arable lands, displaying high growth rates in large-scale photobioreactors or preserving freshwater resources for their cultivation [1, 2]. Bioprospecting efforts have also enlarged the portfolio of marine microalgal species and strains in various culture collections, which constitute important biobanks of potentially bioactive commodities [2]. For example, bioactive extracts have been obtained from several marine microalgae, such as the diatom species model *Phaeodactylum tricornutum*, which contains essential polyunsaturated fatty acids (PUFAs) [3].

Several species of microalgae have been shown to exhibit antioxidant-like activity [4], which can help scavenge reactive oxygen species (ROS) in cells and prevent health-related ailments [5]. ROS-dependent cascades act via the degradation of cellular fatty acids, proteins and nucleic acids and are involved in cell proliferation or cell cycle arrest [6, 7]. There are also indications that some old age-related diseases in humans are associated with decreased resistance to cellular oxidative damage, including Parkinson's disease, Alzheimer's disease, diabetes, hypertension or arthrosclerosis [5, 8–11].

Microalgal metabolites with antioxidant activity include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), lipid peroxidase, mycosporine-like amino acids (MAAs), carotenoid pigments (e.g. fucoxanthin), phytosterols (e.g. campesterol), vitamins (e.g. pro-vitamin), sulphated polysaccharides or flavonoids (e.g. catechin) [5, 12–17]. For example, flavonoids such as kaempferol and quercetin found in *Nannochloris* sp. and *Nannochloropsis* sp. [16] can play an important role in brain function with positive effects on neuronal activity [2, 7, 18]. Microalgae can also produce high-value metabolites such as polyunsaturated fatty acids (PUFAs), especially those of the ω-3 long chain type such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). These have been shown to exhibit bioactivity that may also help with the treatment of conditions such as chronic inflammatory diseases, cancer, diabetes or atherogenesis [18, 19].

The modulation of cultivation conditions in batch culture regimes such as nutrient availability, pH, temperature, light quality and intensity or salinity has been the cornerstone approach by which the yields of particular metabolites have been enhanced for a variety of microalgal species [13, 20, 21]. For instance, changing the environment to which microalgal cells are accustomed can affect their homeostasis and lead them to experiencing oxidative stress. This may be alleviated via the activation of metabolic pathways leading to increasing antioxidant capacity and the accumulation of bioactive compounds [22]. Compared to nutrient concentration manipulation, hydrogen peroxide (H_2O_2) has seldom been considered as a stressor to modulate the homeostasis of microalgae and to influence the production of metabolites, focusing mostly a small number of diatoms and chlorophytes [23–26]. As such, the present study aimed at assessing the physiological responses of marine microalgae species selected from different lineages to H_2O_2-induced oxidative stress in terms of antioxidant activity and proximal biochemical composition as well as the synthesis of high-value pigments and PUFAs.

Material and Methods

Microalgal Strains and Cultivation Setup

Five marine species from distinct microalgal lineages were selected based on their capacity to produce high-value metabolites as established in previous work [27]. These included the dinoflagellate *Amphidinium carterae* LACW11, the rhodophyte *Rhodella violacea* CCAP 1388/5, the haptophyte *Diacronema* sp. GMC45, the diatom *Stauroneis* sp. LACW24 and the chlorophyte *Brachiomonas submarina* APSW11. Each strain was grown in triplicate sterile 1 l Erlenmeyer flasks in a volume of 600 ml in f/2 medium with a 1.5 mg/ml (wet biomass) starting inoculum [28, 29]. The flasks were incubated at 20 ± 1 °C under ca. 60–80 μmol m^{-2} s^{-1} white fluorescent illumination and a 14:10 light:dark photoperiod. The growth was monitored every 4 days by spectrophotometry in 96-well plates at 600 nm using a FLUOstar OMEGA (BMG LABTECH, Ortenberg, Germany).

On day 25, 300 ml of each culture was placed into new flasks in which 0.5 mM H_2O_2 (final concentration) was subsequently added, which based on a pre-screening trial and concentrations previously used in the literature [29, 30]. The original flasks were used as controls. On day 30, the cells were harvested by centrifugation at 2,000 rpm for 5 min to collect the biomass, which was then desalted with 1 ml of 0.5 M ammonium formate prior to overnight freeze-drying (ScanVac, MillRock, Kingston, NY) and subsequent storage at – 20 °C.

Fourier-Transformed Infrared (FTIR) Spectroscopy

FTIR analysis was performed according to McGee et al. [31] using 1 mg of freeze-dried biomass ($n = 3$). The spectra were obtained using a PerkinElmer Model Spectrum Two spectrometer equipped with a diamond crystal attenuated total reflectance cell with a DTGS detector scanning and the wavelength range of 4000–450 cm^{-1} with a resolution of 4 cm^{-1}, and data were acquired with the software Spectrum (version 6, PerkinElmer, Germany). Wavenumber bands were used to determine lipids (C = O ester stretching at 1749 cm^{-1}), carbohydrates (C–O–C stretching at 991 cm^{-1}) and proteins (C = O amide II band at 1703 cm^{-1}) based on the analysis of palmitic acid, starch and bovine serum albumin powders [31–33]. Results were expressed relative to the control samples.

Extract Preparation and Spectrophotometry-Based Assays

Folin–Ciocalteu (FC) Assay

A 5 mg ($n = 3$) of freeze-dried biomass was extracted based on Archer et al. [14] using 1 ml of ethanol (50%) for 20 min at room temperature (20 °C). Samples were vortexed for 30 s and then centrifuged at 10,000 rpm for 4 min; the supernatants were recovered in 1.5 ml tubes. The extraction was repeated with 1 ml of 50% of ethanol, and the two supernatant fractions were combined.

The same extract was then used for carrying out the Folin–Ciocalteu, TEAC and DPPH assays. For the former, 0.1 ml of extract was added to 500 µl of 1:10 diluted Folin–Ciocalteu reagent (Merck KGaA, Darmstadt, Germany) to a 1 ml plastic cuvette. After 4 min, 400 µl of saturated sodium carbonate (37.5 g/l) was added and incubated a second time for 4 h in darkness. Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was used for the calibration curve (0.5–50 µM). The absorbance of samples and controls was measured using a UV–Vis spectrophotometer at 760 nm.

Trolox Equivalent Antioxidant Capacity (TEAC) Assay

The ABTS + radical cation was generated by a reaction of 7 mM ABTS with 2.45 mM potassium persulphate based on Archer et al. [14]. The reaction mixture kept in darkness was used within 2 days. The ABTS + solution was diluted with ethanol (50%) to reach an absorbance of 0.700 ± 0.050 at 734 nm. A 100 µl of ethanolic extract was mixed with 900 µl of diluted ABTS + solution, and the samples were incubated for 15 min at room temperature. The absorbance of samples and controls was immediately recorded at 734 nm. Trolox (0.5 to 20 µM) was used to construct a calibration curve.

DPPH Assay

A 0.25 mM solution of DPPH in methanol was prepared, and a 900 µl of this solution was added to 100 µl of ethanolic extract. After incubation in the dark for 30 min, the absorbance of samples and controls was measured using UV–Vis spectrophotometry at 515 nm [34]. Trolox (0.5 to 300 µM) was used to construct a calibration curve.

Thiobarbituric Acid Reactive Substance (TBARS) Assay

For the lipid peroxidation assay, 5 mg ($n = 3$) of freeze-dried biomass was homogenised in 2 ml of 80:20 (v:v) ethanol:water by vortexing for 1 min, followed by centrifugation at 2,000 rpm for 10 min and collection of the supernatant [34]. Lipid peroxidation was determined in terms of malondialdehyde (MDA) levels within the range of 0.025 to 0.800 mg/ml. A 1 ml aliquot of microalgal extract was mixed with 1 ml of thiobarbituric acid (TBA) solution comprised of 20% (w/v) trichloroacetic acid, 0.01% butylated hydroxytoluene and 0.65% TBA. Samples were then mixed vigorously, heated at 80 °C for 20 min, cooled and centrifuged at 10,000 rpm for 10 min. The absorbance of samples and controls was measured at 532 nm.

Pigment Extraction and Analysis by HPLC–UV-DAD

Extractions were carried out under subdued light from 1 mg ($n = 3$) of freeze and dry biomass using 0.9 ml of ice-cold acetone (100% v/v) ($n = 3$). Afterwards, samples were ground with glass beads in a FastPrep® FP120 for 40 s at a speed of 4.0 $m \cdot s^{-1}$. Before injection, samples were diluted in 90% acetone v/v using dH2O (ultrapure grade) and filtered through with 0.22 µm Whatman filters and stored in amber vials at −20 °C prior to analysis [27].

All HPLC analyses were performed with a Varian ProStar HPLC binary solvent delivery system equipped with ProStar 310 UV and 335 PDA detectors.

The mobile phase and elution gradient program employed was as described in McGee et al. [27]. Pigment identification was achieved by comparing retention times and UV–Vis spectral fine structures to pigment standards (DHI), in-house pigment library [22] and reference data sheets [35].

The quantification of fucoxanthin, β-carotene, peridinin and chlorophyll a and b was performed through reverse phase monolithic column, Merck Chromolith® Performance RP-18 100 × 4.6 mm ID with a stepped gradient solvent program. The quantification of lutein and zeaxanthin was undertaken using a Waters Spherisorb S5 ODS2 4.4 × 250 mm non-end-capped C_{18} column using the mobile phase and elution gradient of McGee et al. [31]. All mobile phases were sonicated and prepared using HPLC HiPerSolv CHROMANORM® grade (methanol, acetone, hexane, acetonitrile and ammonium acetate), and pigments standards were procured from DHI (Denmark) and Sigma.

Lipid Extraction and FAME Analysis by Gas Chromatography-Mass Spectroscopy (GS-MS)

Extractions were performed based on Ryckebosch et al. [36] and Archer et al. [14] using 2.5 mg ($n = 3$) of freeze-dried biomass which was incubated with 0.4 ml of methanol, 0.2 ml of chloroform and 0.04 ml dH_2O. After 30 s of vortex, samples were centrifuged at 2,000 rpm for 4 min. The lower fraction was processed with anhydrous sodium sulphate to remove any residue of water and then dried under a nitrogen stream. The extraction was repeated two more times.

FAME analysis was performed by GC–MS using a BPX70 120 m column with an internal diameter of 0.25 mm on an Agilent7890A/5975C GC–MS system using the ChemStation software version 9.03 (Agilent, Waldbronn). Extracts were re-suspended in 0.25 ml of chloroform (GC–MS grade); 0.1 ml of this extract was then incubated with 0.15 ml of TMSH® (Sigma) for 24 h before injection at a 1:1 split ratio, and an inlet temperature of 250 °C with a helium flow rate of 2 ml/min and the transfer line at 280 °C. The oven program was set up according to Archer et al. [14]: 2 min at 50 °C, ramping at 20 °C/min to 16 °C for 8 min and then 4 °C/min up to 220 °C held for 5 min, with a final step of 4 °C/min to 240 °C and held for 12.5 min.

Data Treatment

Student *t* tests (2-tailed) were carried out to compare the average yields of biomass and relative proportions of fatty acids between the control and H_2O_2 treatments for each microalgal species. One-way ANOVAs using unequal variance assumptions were also used to compare the average antioxidant activities and individual pigment yields between the microalgae. Finally, a principal component analysis (PCA) was also conducted on the data sets to assess the relationships among some of the parameters measured for the microalgae analysed. The PCA was run for a correlation matrix in a varimax rotation mode. The data matrix (eleven variables by ten cases) was transformed by column standardisation prior to analysis. All statistical treatments were performed using IBM SPSS Statistics 26 package 2.

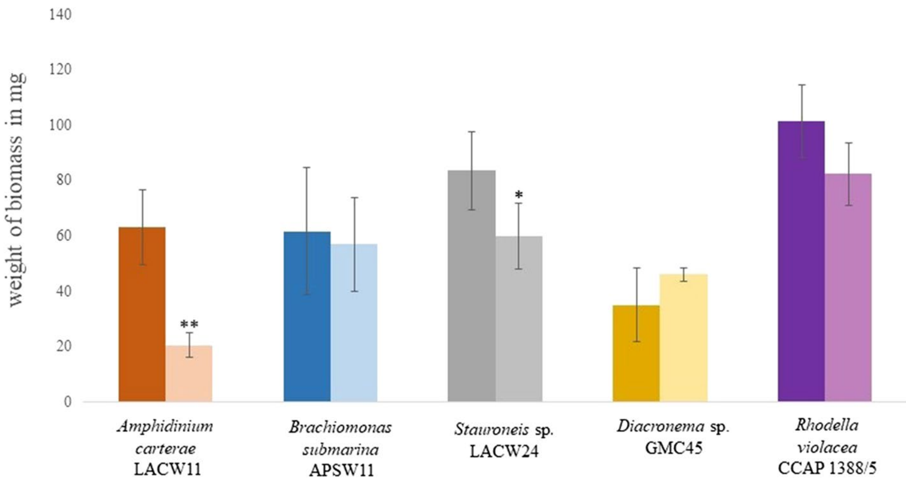

Fig. 1 Comparative yields of biomass (after freeze-drying) retrieved at day 30 by centrifugation from the flasks of the five marine microalgal species cultivated in the absence (dark colour) and presence (light colour) of 0.5 mM H_2O_2. Data are expressed as mean values ± StDev ($n = 3$). ** and * indicate significant differences at $p < 0.01$ and $p < 0.05$, respectively

Results

Particulate Biomass Yields

The addition of H_2O_2 caused an apparent reduction, on average, in the amount of biomass that could be collected by centrifugation from the cultivation tubes (Fig. 1). This was most significant for *A. carterae* with a threefold reduction to 20.6 mg (Student *t*

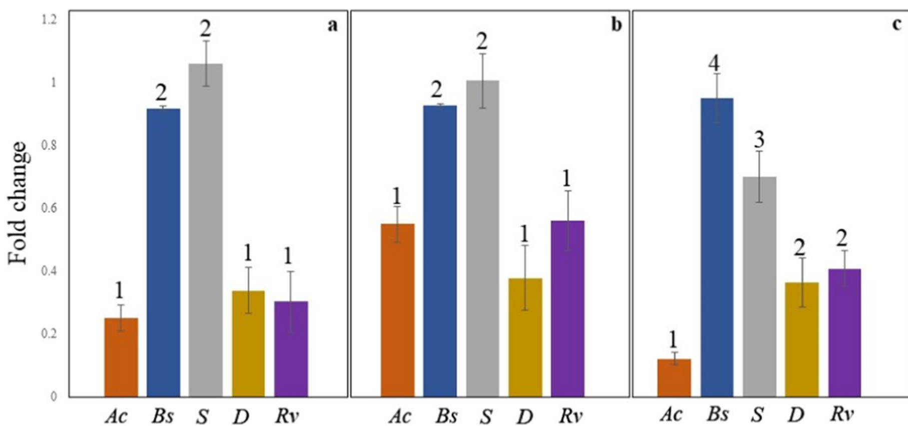

Fig. 2 Comparative fold variation, relative to the controls, in the carbohydrate (**a**), lipid (**b**) and protein (**c**) peak heights of the five microalgal species exposed to 0.5 mM H_2O_2. *Ac*, *Amphidinium carterae* LACW11; *Bs*, *Brachiomonas submarina* APSW11; *S*, *Stauroneis* sp. LACW24; *D*, *Diacronema* sp. GMC45; and *Rv*, *Rhodella violacea* CCAP1388/5. Data are expressed as mean values ± StDev ($n = 3$). Numbers indicate homogeneous subsets ($p < 0.05$)

test, $p < 0.05$). Overall, the rhodophyte *R. violacea* returned significantly more biomass at the end of the experiment (ANOVA, $F = 9$, $p < 0.001$).

Fourier-Transformed Infrared (FTIR) Spectroscopy

FTIR analysis showed significant reductions in the carbohydrate, protein and lipid contents of *A. carterae*, *Diacronema* sp. and *R. violacea* relative to their control treatments (Fig. 2) (ANOVA, $F < 100.3$, $p < 0.05$). Such variation was not as prominent for *B. submarina* and *Stauroneis* sp.

Spectrophotometry-Based Assays

Figure 3 summarises the antioxidant responses observed for the microalgae species tested in the presence and absence of H_2O_2. Overall, lower activities were recorded across the assays for the treatments in which the stressor was added, except for *R. violacea* (Student *t* test, $p < 0.001$). *R. violacea* returned significantly higher values (185 and 210 μmol Trolox eq/g DW under control and H_2O_2 condition, respectively) compared to the other strains for the FC assay (ANOVA, $F = 131$, $p < 0.001$). It also returned the highest antioxidant response with the TEAC assay when exposed to H_2O_2 (13.6 to 42.6 μmol Trolox eq/g DW). However, its responses with the DPPH and TBARS assays were the lowest. The latter assay showed distinct responses among the five microalgae species tested. Notably, significantly greater levels of malondialdehyde were observed in the control treatment for *A. carterae* and the stress treatment for *Diacronema* sp. (ANOVA, $F = 46$, $p < 0.001$).

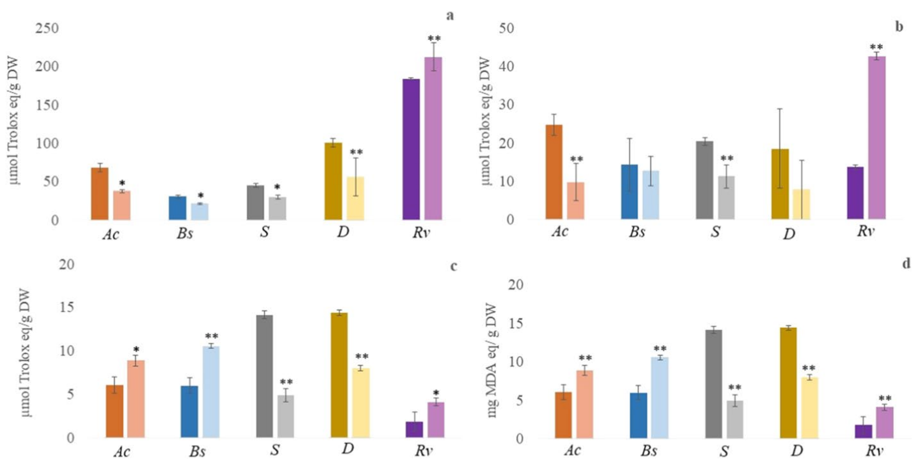

Fig. 3 Comparative responses of the five microalgal species cultivated in the absence (dark colour) and presence (light colour) of 0.5 mM H_2O_2 for the FC (**a**), TEAC (**b**), DPPH (**c**) and TBARS (**d**) assays. *Ac*, *Amphidinium carterae* LACW11; *Bs*, *Brachiomonas submarina* APSW11; *S*, *Stauroneis* sp. LACW24; *D*, *Diacronema* sp. GMC45; and *Rv*, *Rhodella violacea* CCAP1388/5. Data are expressed as mean values ± StDev ($n = 3$). ** and * indicate significant differences at $p < 0.01$ and $p < 0.05$, respectively

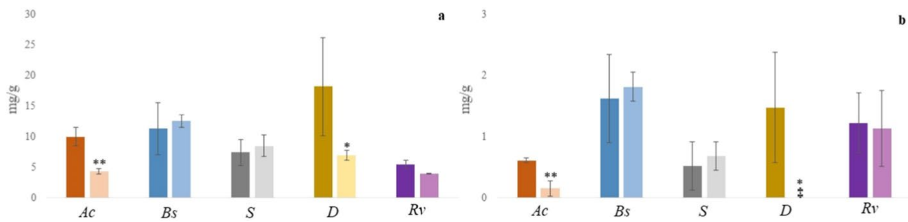

Fig. 4 Comparative yields of chlorophyll a (**a**) and b-carotene (**b**) in the five microalgal species cultivated in the absence (dark colour) and presence (light colour) of 0.5 mM H_2O_2. *Ac*, *Amphidinium carterae* LACW11; *Bs*, *Brachiomonas submarina* APSW11; *S*, *Stauroneis* sp. LACW24; *D*, *Diacronema* sp. GMC45; and *Rv*, *Rhodella violacea* CCAP1388/5. ‡ indicates non-quantifiable response. Data are expressed as mean values ± StDev ($n = 3$). ** and * indicate significant differences at $p < 0.01$ and $p < 0.05$, respectively

Pigment Analysis by HPLC–UV-DAD

The physiological response of the strains to oxidative stress was assessed in relation to pigments. The H_2O_2 treatment typically caused a yield reduction of the major pigments in the strains tested (Fig. 4).

Chlorophyll a was detected in all the extracts, significantly more so for *Diacronema* sp., reaching 18.1 mg/g (ANOVA, $F = 7$, $p < 0.005$), while the chlorophyte *B. submarina* for the H_2O_2 treatment showed a doubling in chlorophyll b (up to 30.3 mg/g). β-carotene was also present in the extracts of the five strains tested, less in *A. carterae* and *Stauroneis* sp., but reaching up to 1.8 mg/g in *B. submarina* (ANOVA, $F = 5$, $p = 0.001$). Other carotenoids showed different responses to the H_2O_2 treatment. There was a 15-fold decrease in peridinin from 2.1 to 0.1 mg/g in *A. carterae* and significant reductions in the fucoxanthin yield of *Diacronema* sp. (3.8 to 0.3 mg/g) and lutein/zeaxanthin yield of *B. submarina* (1.3 to 0.5 mg/g) (Student *t* test, $p > 0.05$). However, H_2O_2 did not cause significant variations in the yields of fucoxanthin in *Stauroneis* sp. (0.7 mg/g) and zeaxanthin in *R. violacea* (0.8 mg/g).

FAME Analysis by Gas Chromatography-Mass Spectroscopy (GS-MS)

The FAME profile of the strains was determined for both the control and H_2O_2 treatments (Fig. 5). C16 and C18 were common to the five species analysed.

Under oxidative stress, *A. carterae* showed a dramatic reduction in C18 proportions (22 to 4%) concomitant to increases in C16, C16:3 and C18:1 (Student *t* test, $p < 0.001$). Compared to the control set, the H_2O_2 treatment caused in the chlorophyte *B. submarina* 10% and 5% increases in the relative proportions of C18 and C18:3, respectively, concomitant to decreases in the proportions of C20:5 (Student *t* test, $p < 0.001$). In *Stauroneis* sp., cell exposure to H_2O_2 caused an increase in the proportions of C18 (24%) (Student *t* test, $p < 0.05$). The H_2O_2 treatment showed for *Diacronema* sp. sharp increases in the proportions of C16 and C18 (32% and 20%, respectively), which were associated with decreases in C18:n (Student *t* test, $p < 0.05$). *R. violacea* showed an alteration of its FAME profile upon exposure to H_2O_2, the proportions of C16 decreasing from 50 to 30%, while C18 and C20:5 increased from 21 to 35% and 15 to 18%, respectively (Student *t* test, $p < 0.01$).

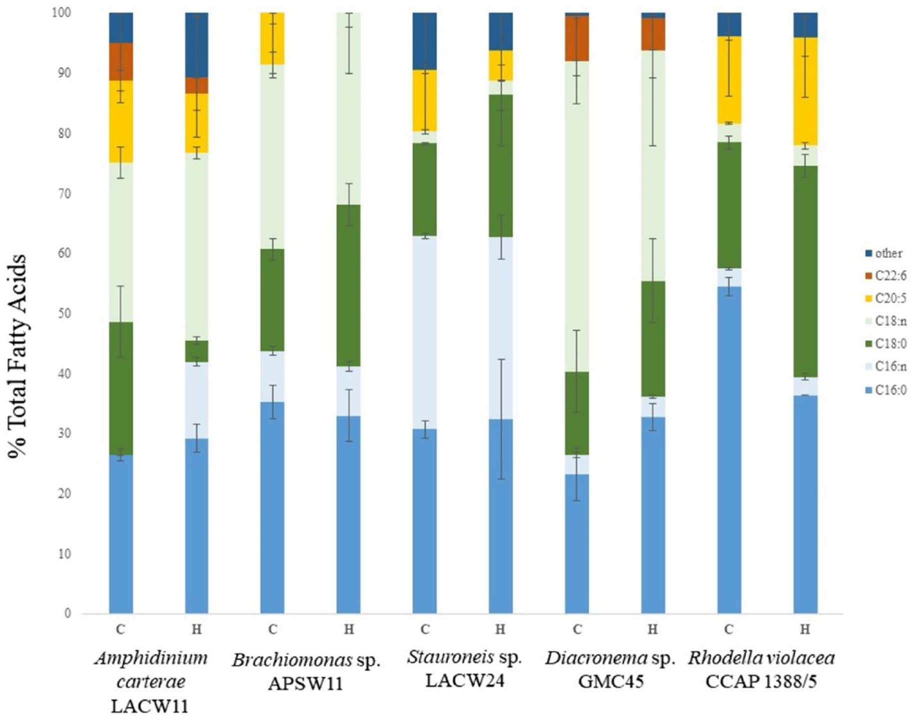

Fig. 5 Comparative proportions of FAMEs in extracts of the five microalgal species cultivated in the absence (C) and presence (H) of 0.5 mM H_2O_2. Data are expressed as mean values ± StDev ($n=3$)

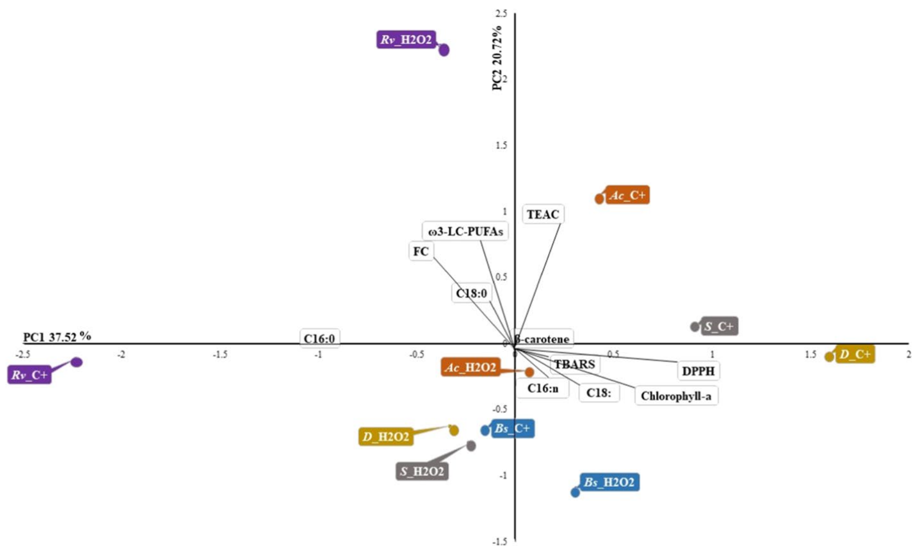

Fig. 6 PCA analysis indicating the relationship between the variables measured (as vectors, with here, proportions of C16:0, C18:0, C16:n, C18:n, ω3-LC-PUFAs, chlorophyll a and β-carotene yields, and the spectrophotometric FC, TEAC, DPPH and TBARS assays) and the projections of the species treatments. *Ac*, *Amphidinium carterae LACW11*; *Bs*, *Brachiomonas submarina APSW11*; *S*, *Stauroneis* sp. *LACW24*; *D*, *Diacronema sp. GMC45*; and *Rv*, *Rhodella violacea CCAP1388/*. C+, controls; H_2O_2, stress condition

The H_2O_2 treatment caused a significant reduction in the proportions of EPA and/or DHA for *A. carterae*, *B. submarina* and *Stauroneis* sp. (Student *t* test, $p < 0.01$).

Principal Component Analysis

The principal component analysis ordination returned 2 components that accounted for 58% of the total variance (Fig. 6). Component 1 (PC1) was strongly positively related to the variables DPPH and chlorophyll a and negatively related to C16:0. Component 2 (PC2) showed a positive correlation between FC, ω3-LC-PUFAs and TEAC. The projection of the treatments in the PCA plane interspersed the species in several zones. Notably, the positions of the control (greater C16 proportions) and H_2O_2 (enhanced EPA and antioxidant activity) treatments for *R. violacea* were clearly isolated on the negative and positive axes of PC1 and PC2, respectively. The H_2O_2 treatments for *Stauroneis* sp., *A. carterae*, *Diacronema* sp. and *B. submarina*, which returned overall low antioxidant responses, were grouped together along the negative domain of PC2, while the corresponding control sets were placed in the PC1–PC2 positive quadrant, except for *B. submarina*.

Discussion

Microalgae have increasingly been considered as sustainable sources of bioactive compounds with applications in the nutraceutical, pharmaceutic, cosmetic, nutrition, biofuel or bio-fertiliser sectors. There is abundant evidence in the literature highlighting that the manipulation of the environment of microalgal cells can influence their homeostasis. This can lead them on occasion to experience stress, which can cause the activation of metabolic pathways promoting the synthesis of bioactive compounds and an increase in their antioxidant capacity [20, 37, 38]. As such, the present study aimed at comparing the responses of several microalgal species from distinct lineages to exposure to H_2O_2-induced oxidative stress. H_2O_2 is a natural product of the catabolism in cells and is naturally present in seawater, varying between 21 and 123 nM along the meridional transect in the eastern Atlantic Ocean [39]. Preliminary trials with the five strains were carried out in 24-well plates using a H_2O_2 concentration range of 0 to 10 mM. They showed the cultures to remain alive when exposed for 15 days to a 0.5 mM concentration (data not shown) even though this level has previously proved toxic toward bacteria and human cells [37]. The scaled up experiment undertaken in flasks of larger volume did not return major differences in the biomass that was retrieved by centrifugation at the end of the incubation period between the control and H_2O_2 treatments, except for the dinoflagellate *A. carterae*, for which a very significant reduction was noted, suggesting cell lysis to have occurred. Similarly, a biomass decrease was previously observed for the freshwater chlorophyte *Monoraphidium* sp. exposed to varying H_2O_2 concentrations (0.5 to 4 mM) [29]. The model species *Phaeodactylum tricornutum* also showed a significative reduction in biomass from exposure to 0.25 to 2 mM of H_2O_2, which has been suggested as a potential trigger of apoptosis in diatoms [26, 40]. Another study demonstrated for the ochrophyte *Aureococcus anophagefferens* that cell size is a key factor to H_2O_2 sensitivity, which involves reactions with a wide range of cellular organic compounds such as alcohols, esters or aromatics [39]. The effects of H_2O_2 were also visible from the FTIR analyses, which showed overall reductions in proteins, carbohydrates and lipids compared to the control flasks for *A. carterae*, *Diacronema* sp. and *R.*

violacea, corroborating the fact that microalgae show variations in cellular yields of some macromolecules when exposed to stressing environments [41].

The FC, TEAC, TBARS and DPPH assays are based on spectrophotometry methods that have been applied for determining the antioxidant potential of extracts from a variety of biological matrices, including microalgae, and have proved popular in the context of food science and health nutrition [42–44]. Several studies have indicated that different solvents and solvent strengths can lead to the preferential extraction of compounds, which can return differential antioxidant potential estimation using different assays [45–47]. Carrying out multiple extractions for several assays can require a substantial amount of biomass being used, which can be limiting, especially when multiple experimental parameters are being tested. Here, in order to minimise the amount of biomass needed and to compare the effects of the generated extracts between the assays used, a single solvent system (50% ethanol) was chosen for assessing the antioxidant activity of the microalgal extracts. Overall, the antioxidant activity values obtained in the present study were largely aligned with those reported in the literature, the FC and TEAC assays having been previously used to analyse Rhodophyceae (16.6–67.9 μmol Trolox eq/g DW), Dinophyceae (2.2–6.3 μmol Trolox eq/g DW), Pavlophyceae (24.2–94.2 μmol Trolox eq/g DW), Chlorophyceae (5.5–214.3 μmol Trolox eq/g DW) and Bacillariophyceae (4.5–48.9 μmol Trolox eq/g DW) species [5, 42, 48–51]. Exposure to 0.5 mM H_2O_2 caused an overall decrease in the antioxidant response of the microalgae species tested, except for *R. violacea*. Some species-specific response patterns were however observed for some assays; for example, *A. carterae* and *B. submarina* returned higher responses for the DPPH assay when exposed to H_2O_2. *R. violacea* also showed a very weak TBARS response compared to the other species. Interestingly, the TBARS responses of *Stauroneis* sp. and *Diacronema* sp., which are more phylogenetically related to one another than the three other species, were higher for the controls than treatments. It was previously shown in diatoms that the levels of malondialdehyde might not increase upon stress exposure due to the production of fucoxanthin or other antioxidant compounds [52, 53]. Here, a significant reduction in fucoxanthin was observed in *Diacronema* sp., which would suggest the prevalence of an enzymatic response to counteract the effects of H_2O_2.

Pigment analysis showed the typical signatures expected of the microalgal species used in this study [35]. Exposure of the cells to 0.5 mM H_2O_2 caused significant reductions in the chlorophyll a and β-carotene contents of *Diacronema* sp. and *A. carterae* as well as fucoxanthin in *Diacronema* sp and lutein in *B. submarina*. However, H_2O_2 enhanced chlorophyll b levels in *B. submarina.* Reactive oxygen species (ROS) have been described as key compounds responsible for the degradation of photosystems I and II in microalgae, which can also lead to concomitant biomass reduction as ROS can trigger autophagy in plants and algae [54]. H_2O_2 is a stable ROS that can diffuse through the membranes of chloroplasts [21]. In this context, chlorophyll quota increases have been proposed as mechanisms of protection of chloroplasts against ROS damage [55]. As such, previous work on the freshwater *Chlorella vulgaris* showed an increase in chlorophyll after exposure to 0.5% H_2O_2 [47]. This was also observed for *Raphidocelis subcapitata* exposed to ROS evoked by erythromycin as well as *Microcystis aeruginosa* subjected to the antibiotic enrofloxacin [55, 56]. The Chlorophyll b content was also enhanced in *Aerococcus anophagefferens* after incubation with H_2O_2 [39].

The homeostasis of cells partially depends on the capacity of cellular antioxidant response mechanisms to cope with oxidative stress. Carotenoid pigments contribute to help protecting, along with the enzymatic-based response, against oxidative stress triggered by various factors [57]. In the present study, the yields of the xanthophyll fucoxanthin in

Stauroneis sp. and zeaxanthin in *R. violacea* were not significantly affected by the H_2O_2 treatment, possibly suggesting that the antioxidant response had not been overwhelmed for these two species.

The exposure of the five microalgal species to H_2O_2 triggered variation in their fatty acid signatures. Notably, C16:n and C18:n fatty acids were enhanced in *A. carterae*, while the proportions of C18 increased for the four other species. The proportions of the PUFAs EPA and/or DHA also decreased for *B. submarina*, *Stauroneis* sp. and *Diacronema* sp. *R. violacea* showed however increases of both C18:0 and EPA, the former being an important precursor for the biosynthesis of LC-PUFAs in microalgae [58]. Isoprostanoids and oxylipins, which correspond to oxidised PUFAs, have attracted enhanced interests due to their cell signalling functions and potential beneficial effects [59]. It was recently shown that H_2O_2 can trigger the production of isoprostanoids derived from C18, C20 and C22 in the marine diatom *Phaeodactylum tricornutum*, which may explain the EPA proportion reduction observed in *Stauroneis* sp. [59–61]. The response patterns were overall species-specific, which was also reflected in the relative positions of the treatments along the axes delineated by the PCA planes. Other studies have documented the effects of H_2O_2 on lipids in microalgae. For example, the content of neutral lipids, both at early and late stationary phases, was enhanced by ~ 30% (up to 96 mg/L) in *P. tricornutum* exposed to 0.25 mM H_2O_2 [26]. In this context, Qiao et al. [26] recently highlighted that the combination of NaCl and H_2O_2 stress in the freshwater *Monoraphidium* sp. can positively influence the expression of the lipogenesis regulator genes accD, KASIII and DGAT1. Overall, the present study, carried out with members of different microalgal lineages, confirmed that H_2O_2 can be utilised as an effective stressor to influence the homeostasis of microalgae. However, species-specific responses were observed with regard to the expression of different types of metabolites and antioxidant activity, which may reflect differential sensitivities to similar levels of H_2O_2. Further investigations could be warranted, especially with rhodophytes, given the overall positive response the H_2O_2 treatment elicited in *Rhodella* in terms of enhanced EPA content and antioxidant activity.

Conclusion

The commercial exploitation of microalgal bioactive compounds is currently challenged by production and processing costs, requiring the optimisation of cultivation regimes and enhancement of cellular yields of high-value compounds. In this study, exposure of five marine microalgae to H_2O_2 stress, which can promote the synthesis of particular metabolites, interestingly showed species-specific responses. Notably, *R. violacea* showed enhanced antioxidant activity (> twofold for TEAC and DPPH assays) and EPA proportions, while the other species showed reductions in chlorophyll a and β-carotene and varying patterns of fatty acid profiles. Results indicated that exposure of microalgae cells to H_2O_2 can initiate distinct metabolic changes in different species, which would require further optimisation of the dosage and cultivation conditions to modulate the yields of specific metabolites in individual species.

Author Contribution MEB, GTAF and NT developed the experimental design. MEB conducted all experiments and analyses and led the drafting of the manuscript. RP, DF, AA, TC, HH and AMD provided technical support and helped with the FTIR, AOX, HPLC and GC–MS methods. All authors contributed to editing and finalising the manuscript.

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Declarations

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