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Combined nitrogen limitation and hydrogen peroxide treatment enhances neutral lipid accumulation in the marine diatom *Phaeodactylum tricornutum*

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ABSTRACT

Exogenous application of dilute hydrogen peroxide (H_2O_2) increases neutral lipid production in *Phaeodactylum tricornutum*. Exposing early stationary phase cultures of *P. tricornutum* to 0.25 – 2 mM H_2O_2 increases the amount of neutral lipids per biomass (mg/mg) by >100% at 24 hours post H_2O_2 treatment as determined upon lipid extraction and analysis using a neutral lipid assay. H_2O_2 treatment increased the total levels of neutral lipids harvested up to 50%, from 64 mg/L to 96 mg/L, demonstrating its possible effectiveness as a pre-harvest strategy to enhance the biofuel feedstock potential of *P. tricornutum*. The effects of H_2O_2 on biomass are concentration dependent; increasing concentrations of H_2O_2 reduce the levels of isolated biomass. Analysis of combined stressors demonstrates that H_2O_2 treatment exhibits synergistic effects to enhance neutral lipid production under nitrogen-depleted, but not phosphorus-depleted conditions, suggesting that the effects of hydrogen peroxide on lipid production are influenced by environmental nitrogen levels.

KEY WORDS

Phaeodactylum tricornutum, Oxidative stress, Nitrogen limitation, Lipid accumulation, Hydrogen peroxide

1. INTRODUCTION

Neutral lipids derived from microalgae cultivation show promise as a feedstock for biofuel production. Specific microalgae species contain high amounts of the neutral lipid class triacylglycerol (TAG), have fast growth rates, and are an attractive alternative for feedstock production versus plant-based systems (Chisti, 2007; Mata et al., 2010).

Although the production of commercial biodiesel derived from microalgae oil has been achieved, the cost is not currently competitive with fossil fuel (Jones & Mayfield, 2012; Nagarajan et al., 2013). For microalgae to become economically viable as a biofuel feedstock, it is necessary to understand and develop conditions that enhance lipid production such as late-stage, pre-harvest treatments that can increase lipid production.

Abiotic stress is known to enhance microalgae lipid production (Hu et al., 2008). Although abiotic stressors often negatively affect microalgae growth rates and decrease biomass productivity (Pancha et al., 2015; Xin et al., 2011), inducing stress can be an effective strategy to enhance lipid production if there is an overall increase in lipids after compensating for biomass loss. The most common abiotic stress used to enhance lipid production in microalgae is nitrogen starvation. During periods of limited nitrogen availability, microalgae undergo a rapid cellular and metabolic remodeling process that enhances their ability to survive following recovery from the stress. These changes include modification of central metabolic pathways, increased expression of nitrogen transport and assimilation proteins and genes, as well as a general down regulation of photosynthetic activity (Guerra et al., 2013; Liang et al., 2013; Valenzuela et al., 2012; Wase et al., 2014; Yang et al., 2013). Global gene expression analyses of microalgae following nitrogen

deprivation have also identified key pathways and transcription factors responsible for a shift toward neutral lipid production (Boyle et al., 2012; Li et al., 2014; Wang et al., 2014).

Markers of oxidative stress, such as increases in antioxidant expression and lipid peroxidation, are often detected during episodes of abiotic stress in microalgae. Nitrogen deprivation (Yilancioglu et al., 2014; Zhang et al., 2013), salinity (Pancha et al., 2015), temperature (Xin et al., 2011) and light stress (Menon et al., 2013) are all linked to an increase in reactive oxygen species (ROS) or oxidative stress levels in parallel with enhanced lipid accumulation observed during the stress. Direct application of hydrogen peroxide (H_2O_2), which is a naturally occurring ROS produced by cellular processes, has been shown to enhance lipid accumulation in microalgae (Battah et al., 2014; Yilancioglu et al., 2014; Zhang et al., 2013), providing further support for the connection between oxidative stress and intracellular lipid accumulation.

This study is the first to describe the effects of hydrogen peroxide to increase lipid accumulation in diatoms. Diatoms are a diverse and ecologically important group of organisms that play important roles in global carbon fixation and global biomass production (Lebeau and Robert, 2003). Select diatoms species are considered as attractive candidates for biofuel feedstock production based on their fast growth rates and the ability to accumulate neutral lipids under abiotic stress conditions such as nitrogen- and silica-deprivation (d'Ippolito et al., 2015; Hildebrand et al., 2012). *P. tricornutum* has been investigated for its potential application in bioenergy production, in aquaculture, and for producing large amounts of the omega-3 fatty acid eicosapenaeic acid (EPA) (Lebeau and Robert, 2003).

This study reports the temporal and concentration-dependent effects of peroxide-induced oxidative stress on neutral lipid accumulation in diatoms. This study also describes the effect of combining abiotic stress and report the novel finding that peroxide-induced neutral lipid production occurs only under low environmental nitrogen levels. The effects of combining abiotic stress are not general: enhanced neutral lipid accumulation was not observed when peroxide treatment was combined with phosphorus-depletion conditions. Due to the relative safety, simplicity and reasonable cost associated with the treatment of microalgae cultures with dilute H₂O₂, we propose that H₂O₂ treatment can be developed into a pre-harvest strategy of relevance to the algae biofuel industry.

2. MATERIALS AND METHODS

2.1 Microalgae culture conditions

Axenic cultures of *P. tricornutum* (UTEX 466) were obtained from UTEX (University of Texas, Austin, TX) and maintained on agar plates made of F/2 media (NCMA, East Boothbay, ME). For experiments, *P. tricornutum* colonies were inoculated into F/2 liquid culture in 2-L Corning Pyrex media bottles (Corning 1395-2L) using stir bars with air bubbling (Petco Air Pump). Incoming air is sterile filtered by Polyvent 4 disposable filters (Whatman, Kent, UK). The culture suspensions were maintained at a constant temperature of 23 ± 2 °C with full spectrum incident uniform lighting (High Efficiency T-5 Grow Lights – Gardeners Supply Co, VT) at a 16:8 h light/dark cycle (60-120 M photons/m/s). All equipment and materials were autoclaved and media added to flasks in a Labconco Purifier Vertical Clean bench (Labconco, Kansas City, MO). During culturing, cell density was measured every other day at 680 nm using a Thermo Scientific Genesys 10S Vis Spectrophotometer (ThermoFisher, San Jose, CA).

2.2 Microalgae treatment with hydrogen peroxide

Liquid stock cultures of *P. tricornutum* were grown to early stationary phase (approximately 8-12 days) based on absorbance measurements at 680 nm. Culture aliquots of 149 mL (or 199 mL) were transferred to 500-mL Corning Pyrex media bottles (Corning 1395-500) prior to H₂O₂ treatment. Culture volumes depend on number of experimental replicates used during treatment. An aqueous solution of 30% hydrogen peroxide (9.79 M, Thermo Fisher, San Jose, CA) was further diluted in H₂O to prepare working stock concentrations of H₂O₂ (e.g. 0.038 mM, 0.11 mM and 0.30 mM for 150 mL experiments). Following dilution, 1.0 mL of each working stock solution was added to the corresponding culture flask to produce final H₂O₂ concentrations ranging from 0.25 mM to 20 mM in each *P. tricornutum* culture. All experiments were performed in triplicate (n = 3) and compared to untreated (H₂O) controls with 1 mL of H₂O added in place of the 1 mL peroxide stock solution.

2.3 Microalgae treatment with hydrogen peroxide during induced nutrient-depleted conditions

For experiments investigating combined abiotic stress of hydrogen peroxide treatment and nitrogen- or phosphorus-depleted conditions, early stationary phase cultures were centrifuged and re-suspended in 149 or 199 mL of media containing F/2 media with or without supplementation of the recommended concentration of nitrate or phosphate salts as described in the F/2 media recipe. (condition 1: 100% N, 100% P; condition 2: 0% N, 100% P; condition 3: 100% N, 0% P). Experiments investigating re-supplementation with

139 ranges of nitrate salts (5% N, 10% N, 25% N, etc.) were also performed. Following re-
140 supplementation, algae cultures were then transferred to 500-mL Corning Pyrex media
141 bottles as described above. Cultures were grown for 48 h after re-supplementation and then
142 treated with 1 mL of peroxide stock solution to expose cultures to 2 mM H₂O₂ for another
143 24 hours prior to biomass harvesting and lipid analysis. All experiments were performed in
144 triplicate (n = 3) and compared to non-treated H₂O controls.

146 *2.4 MDA measurement using a thiobarbituric acid reactive species (TBARS) assay*

147 A TBARS assay was used to measure changes in malodialdehyde (MDA) levels as a
148 marker of lipid peroxidation levels to approximate changes in oxidative stress.
149 Measurements were performed on microalgae aliquots in microplates using methods similar
150 to those described by Ohkawa and colleagues (Ohkawa et al., 1979). Microalgae aliquots
151 were combined with trichloroacetic acid (TCA) and thiobarbituric acid (TBA) and boiled
152 for 5 minutes. Absorbance readings at 532 nm were recorded on boiled algae samples using
153 a Synergy HT Multi-Mode Plate Reader (Biotek, Vermont, USA). The amount of MDA per
154 sample was determined using a standard curve of known MDA samples. The amount of
155 MDA per sample was normalized based on cell density and presented as fold-change versus
156 untreated (H₂O) controls.

158 *2.5 Biomass determination*

159 At both 3- and 24-hour time points following hydrogen peroxide treatment,
160 microalgae cultures (150 or 200 mL) were pelleted by centrifugation and washed once with
161 H₂O. Algae pellets were then frozen in liquid nitrogen and lyophilized to dryness

(Labconco Freezone 6, Kansas City, MO). Lyophilized biomass weights were recorded and are reported as isolated biomass per liter culture. Lyophilized biomass was stored at –20 °C under argon gas until lipid extraction was performed.

2.6 Lipid extraction

Total lipids were extracted from freeze-dried algae biomass using a modified Folch method (Folch et al., 1957). Microalgae samples were first treated with chloroform and pulverized using a hand sonicator (Fisher Scientific Model 120 Sonic Dismembrator, ThermoFisher, San Jose) for 1 minute. Next, a 2:1 mixture of methanol/chloroform was added to samples and sonication repeated. Then 0.1 M PBS was added to samples, mixed vigorously, and centrifuged to separate layers. The lower organic (chloroform) phase containing lipids was removed and stored overnight at –20 °C.

2.7 Neutral lipid content determination using a bleach-enhanced Nile-Red microplate lipid extract assay

A bleach-enhanced Nile red microplate assay (adapted from Higgins et al., 2014). was performed to quantify the amount of neutral lipids in the total lipid extract from microalgae. This quantitative assay compares Nile Red (9-diethylamino-5H-benzo[*a*]phenoxazine-5-one, Sigma, USA) fluorescence of lipid extracts to a standard curve of known corn oil concentrations. This assay is referred to as a Nile Red neutral lipid assay throughout the paper. Lipid extracts in chloroform were diluted 2:1 in methanol and added to a 96-well polypropylene microplate (Evergreen Scientific, Los Angeles, CA) in triplicate. Therefore, neutral lipid analysis utilized three technical replicates for each of the

three biological replicate for all conditions studied ($n = 9$). In one case, an outlier was removed from a dataset and therefore utilized a sample size with $n = 8$ (see Table 1 for details). Corn oil standards were treated similarly and added to the same microplate in quadruplicate to achieve a range of 0 to 40 μg per well. The microplate was heated to 55 $^{\circ}\text{C}$ to evaporate the solvent and, after cooling to room temperature, the extracts were resuspended in isopropyl alcohol to redissolve the lipid sample. The lipid samples were treated with Nile Red in DMSO and H_2O (final concentration of Nile Red $\sim 160 \mu\text{g/mL}$, final concentration DMSO $\sim 0.25\%$ (v/v)) and allowed to incubate in the dark for five minutes. A 3% aqueous solution of sodium hypochlorite (made by 1:1 dilution of Clorox bleach in H_2O) was then added to samples and fluorescence measurements were captured using a Synergy HI Multi-Mode Plate Reader (Biotek, Vermont, USA) at excitation and emission wavelength of 530/40 nm and 590/40 nm, respectively. The neutral lipid content of each lipid extract was quantified using a standard curve derived from corn oil standards.

2.8 TLC analysis of lipid extracts

An aliquot of 30 μL of extracted lipids in chloroform was spotted on glass-backed silica-coated plates (TLG-R10014BK-323, SiliCycle, Canada) and separated using a solvent system of hexanes/diethyl ether/acetic acid (70:30:1, v/v). Separated lipids were visualized by exposure to a 0.75 w/v % aqueous KMnO_4 solution containing 5 w/v % K_2CO_3 and 6.25 w/v% NaOH and then imaged using a Panasonic digital camera (model number DMC-FH25, Japan). An aliquot of 5 μL of 1 mg/mL corn oil in chloroform was

spotted on TLC plates alongside extracted samples to serve a triacylglycerol (TAG) reference.

2.9 Statistical analysis

All results are expressed as mean values $\pm$ standard deviation. Significant differences ($p < 0.05$) in datasets were determined using one-way ANOVA, and post hoc Tukey's HSD tests were used to determine significant differences ($p < 0.05$) within the experimental conditions of a data set.

3. RESULTS AND DISCUSSION

3.1 Effects of hydrogen peroxide treatment on levels of oxidative stress and biomass in *P. tricornutum*

P. tricornutum cultures exposed to hydrogen peroxide in the early stationary growth phase respond in a concentration- and time-dependent fashion (**Figures 1a-c**). Following H_2O_2 application, changes in lipid peroxidation levels were undetectable in cultures exposed to 0.25 mM H_2O_2 conditions, whereas lipid peroxidation increased 4-fold in 0.75 mM H_2O_2 and 13-fold in 2 mM H_2O_2 conditions, as compared to untreated (H_2O) controls when measured using the TBARS assay (**Figure 1a**). No changes in isolated biomass levels were observed 24 hours after culturing in 0.25 mM H_2O_2 when compared to untreated (H_2O) controls (**Figure 1b**). Over the same time period, isolated biomass decreased 59% and 63% with 0.75 mM H_2O_2 and 2 mM H_2O_2 , respectively (**Figure 1b**). Cell density as measured by absorbance (680 nm) further confirmed that exposing cultures

to 0.75 mM H₂O₂ and 2 mM H₂O₂, but not 0.25 mM H₂O₂, negatively affected *P. tricornutum* biomass levels (**Figure 1c**). This data suggests that higher concentrations of H₂O₂ will induce oxidative stress and cause cell death in *P. tricornutum*, which is consistent with recent evidence that implicates H₂O₂ as an inducer of programmed cell death in diatoms (van Creveld et al., 2015).

3.2 Effects of hydrogen peroxide treatment on neutral lipid content in *P. tricornutum*

The concentration and time-dependent effects of H₂O₂ treatment on neutral lipid content for cultures of *P. tricornutum* at early stationary phase was determined using a Nile Red neutral lipid assay on extracted lipid samples (**Figures 2a,b**). After three hours of peroxide exposure, there was no significant difference ($p > 0.05$) in the neutral lipid content of H₂O₂-treated conditions when compared to untreated (H₂O) controls, with neutral lipid contents ranging from 0.19 – 0.23 mg per biomass (mg/mg) (**Figure 2a**). All peroxide conditions showed significant ($p < 0.05$) increases in the amount of neutral lipids extracted per biomass after 24 hours of peroxide treatment, where the neutral lipid content in untreated (H₂O) controls was 0.23 mg/mg, and the neutral lipid content in H₂O₂-treated conditions increased to 0.34 mg/mg (in 0.25 mM H₂O₂), 0.56 mg/mg (in 0.75 mM H₂O₂), and 0.59 mg/mg (in 2 mM H₂O₂) (**Table 1** and **Figure 2a**). Thin-layer chromatography (TLC) confirmed an increase in TAG levels in the neutral lipid profile as a response to H₂O₂ treatment (**Figure 3c**). This demonstrates that H₂O₂ application to *P. tricornutum* cultures at early stationary phase increases the amount of neutral lipids contained in the isolated biomass up to 156% (**Table 1**).

The total amounts of neutral lipids harvested from *P. tricornutum* were analyzed to determine if H₂O₂ application enhanced neutral lipids when considering the productivity of neutral lipids on a per culture basis (mg/L). No significant ($p > 0.05$) change in the quantity of neutral lipids harvested was observed after a 3-hour treatment period. Neutral lipids harvested from *P. tricornutum* after a 24-hour H₂O₂ treatment were 91 mg/L, 94 mg/L and 96 mg/L for 0.25 mM H₂O₂, 0.75 mM H₂O₂ and 2 mM H₂O₂ conditions, respectively (**Table 1** and **Figure 2b**). Compared to 64 mg/L of neutral lipids harvested for the untreated (H₂O) control, the H₂O₂ treatment significantly ($p < 0.05$) increased the total amount of neutral lipids harvested by 42%, 47%, and 50%, respectively. The increase in the total amount of neutral lipid harvested 24-hour after the addition of H₂O₂ shows the potential of H₂O₂ as a pre-harvest treatment in the algae biofuels industry because the increases in neutral lipid content are more significant than any loss in isolated biomass that might result from the treatment conditions. Based on the cost of an aqueous solution of 35% (w/w) hydrogen peroxide at \$42.25/L (Sigma, 349887-4), the total cost of exposing *P. tricornutum* cultures to a concentration of 0.25 mM H₂O₂ is approximately \$0.001/L (0.022 μ L of 35% H₂O₂ required per liter culture) and is expected to lower the unit cost of producing esterified oil from this species.

The mechanism by which H₂O₂ enhances neutral lipid production in microalgae remains unknown, but rerouting metabolic flux has been identified as a key method for mitigating oxidative stress in *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (Ralser et al., 2007). Because fatty acid biosynthesis has a large energy requirement when compared to carbohydrate and protein synthesis, shifting metabolism towards enhanced

neutral lipid biosynthesis during stress may provide protective function by acting as an efficient electron sink to reduce ROS generation (Lemoine & Schoefs, 2010; Solovchenko, 2012). Our results also show a statistically significant ($p < 0.05$) enhancement of neutral lipid production using low concentrations of H_2O_2 (0.25 mM) where biomass levels did not decrease (**Figures 1b and 2, Table 1**). An increase in neutral lipids without a reduction in biomass during a 24-hour exposure to 0.25 mM H_2O_2 suggests that our findings may be partially attributed to the known ability of H_2O_2 to function as an intracellular signaling molecule (Schieber & Chandel, 2014) and that oxidative damage is not solely responsible for the enhanced neutral lipid production observed upon H_2O_2 treatment in *P. tricornutum*. We have previously identified that the addition of small molecules with known oxidative signaling mechanisms can enhance neutral lipids in *P. tricornutum* (Franz et al., 2013). Hydrogen peroxide has also been reported to influence post-translational regulation of key proteins involved in nitrogen assimilation and lipid biosynthesis in *P. tricornutum* (Rosenwasser et al., 2014).

3.3 Effects of nitrogen levels on biomass and oxidative stress levels in P. tricornutum during hydrogen peroxide treatment

Because the transition to stationary phase coincides with nitrogen depletion (Valenzuela et al., 2012), we hypothesized that there may be a synergistic effect between environmental nitrogen levels and the H_2O_2 -induced accumulation of neutral lipids in *P. tricornutum*. Consequently, the combined effects of H_2O_2 treatment during induced nitrogen-depleted conditions was further evaluated to understand the relationship between environmental nitrogen status and oxidative stress induction in *P. tricornutum* (**Figure 3**).

P. tricornutum was grown to early stationary phase and then re-suspended in fresh media supplemented with nitrogen or without nitrogen to expose cultures to either nitrogen-replete (100% N) or nitrogen-deplete (0% N) conditions, respectively. Following resuspension of *P. tricornutum* with new media, cell density (as monitored by absorbance) after 48 hours increased 80% in cultures with new supplies of nitrogen compared to the cell density of cultures with no supplemental nitrogen (**Figure 3a**). After the 48-hour incubation in either nitrogen-replete or nitrogen-deplete conditions, cultures were exposed to 2 mM H₂O₂. The levels of isolated biomass harvested 24 hours after H₂O₂ treatment were decreased for both nitrogen-replete (18%) and nitrogen-depleted conditions (50%) compared to their respective untreated (H₂O) controls (**Figure 3b**). Oxidative stress, as measured using the TBARS assay, increased up to 2-fold in nitrogen-replete cultures and up to 5-fold in nitrogen-depleted cultures following H₂O₂ treatment (**Figure 3c**). Consistent with research in the green algae species *Chlorella sorokiniana* C3 (Zhang et al., 2013), nitrogen depletion alone enhances lipid peroxidation (approximately 2-fold) in diatoms as determined using the TBARS assay (**Figure 3c**).

3.4 Effects of nitrogen and phosphorus on hydrogen peroxide-induced neutral lipid production in P. tricornutum

Environmental nitrogen levels were identified as a determining factor in the effect of hydrogen peroxide to enhance neutral lipid accumulation in *P. tricornutum* (**Figure 4a**). Using a neutral lipid Nile Red assay on extracted lipid samples, the addition of hydrogen peroxide was determined to have no significant ($p > 0.05$) effect on the amount of neutral lipids per biomass (mg/mg) during nitrogen-replete conditions. Under nitrogen-replete

conditions, the neutral lipid content of biomass was 0.06 mg/mg following 24-hour peroxide application versus 0.05 mg/mg observed in H₂O treated controls. Exposure to nitrogen-depleted conditions without H₂O₂ treatment increased neutral lipids per biomass to 0.29 mg/mg. A combination of nitrogen depletion and H₂O₂ treatment demonstrated a synergistic effect where the neutral lipid content increased 92%, from 0.29 mg/mg to 0.56 mg/mg. Subsequent experiments analyzing levels of nitrogen-limitation revealed that peroxide treatment enhanced the neutral lipid content of *P. tricornutum* at 5% and 10% nitrogen supplementation levels but was not observed with 25% nitrogen supplementation (**supplemental Figure 1**).

While these findings demonstrated that low environmental nitrogen levels are required for hydrogen peroxide-enhanced neutral lipid production, it was not known if limiting other nutrients would also exhibit similar synergistic effects. Because phosphorus-limitation is also known to enhance neutral lipid accumulation in *P. tricornutum* (Valenzuela et al., 2012, Valenzuela et al., 2013), the combination of H₂O₂ treatment with phosphorus-depleted conditions was evaluated to investigate if peroxide-enhanced production of neutral lipid occurs as a general response to other types of nutrient-limited conditions.

The combination of peroxide treatment with phosphorus-deplete (0% P) conditions did not increase neutral lipid content in *P. tricornutum*, confirming that the synergistic effect observed with nitrogen-limited conditions is not a general response. Culturing in phosphate-depleted conditions increased the neutral lipid content per biomass (mg/mg) to 0.12 mg/mg versus 0.06 mg/mg observed in phosphate-replete (100% P) conditions (**Figure 4a**). When phosphorus-depletion was combined with hydrogen peroxide

treatment, a decrease in the neutral lipids per biomass (mg/mg) was observed, from 0.12 mg/mg to 0.08 mg/mg (**Figure 4a**). TLC was used as an additional analysis method to further confirm that the observed increase in neutral lipids as a response to hydrogen peroxide treatment in the nitrogen-depleted conditions can be attributed to increasing TAG levels (**Figure 4b**).

Our results demonstrate that nitrogen-limited conditions are required for hydrogen peroxide-induced neutral lipid accumulation in *P. tricornutum*. We observed that peroxide treatment increases neutral lipids in *P. tricornutum* cultures both at early stationary phase and when nitrogen levels were artificially depleted. These results support recent findings that H₂O₂ may be involved in the communication of environmental nitrogen levels in *P. tricornutum* through post-translational regulation of nitrogen assimilation proteins (Rosenwasser et al., 2014). Inhibition of nitrogen assimilation through chemical or genetic manipulation has been observed to increase lipid content in microalgae including *P. tricornutum* and *Chlorella desiccata* (Levitan et al., 2015; Rachutin Zalogin & Pick, 2014). Therefore, H₂O₂ treatment may function by enhancing redox regulation as part of a mechanism that shifts metabolism away from growth and towards lipid production during periods of low environmental nitrogen.

CONCLUSIONS

The addition of dilute hydrogen peroxide to *P. tricornutum* cultures at early stationary phase enhances neutral lipid production. Twenty-four hours after hydrogen peroxide application, the neutral lipid content of biomass increased >100% and the total neutral lipids harvested increased up to 50% when compared to non-treated cultures as

364 controls. This study establishes the effects of combined abiotic stressors, demonstrating that
365 H₂O₂ treatment exhibits synergistic effects for enhancing lipid production at low nitrogen
366 levels. Finally, this study indicates the potential of dilute H₂O₂ application in microalgae
367 cultures as a pre-harvest treatment to boost neutral lipid content for biofuel production.
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FIGURE CAPTIONS

Figure 1: Hydrogen peroxide effects on early stationary phase *P. tricornutum* oxidative stress levels by MDA content (A), isolated biomass (B), and cell density (C). Data shown as mean $\pm$ SD (n = 3).

Figure 2: Effects of hydrogen peroxide on *P. tricornutum* neutral lipid content per biomass (A) and neutral lipids harvested per liter (B). Conditions represent three biological replicates each with three technical replicates (n = 9) per analysis except for one case (0.25 mM H₂O₂, neutral lipids harvested per liter) where one technical replicate was removed from the dataset (n = 8, see explanation in **Table 1**). Conditions denoted with similar letters did not differ significantly (p < 0.05) (ANOVA, Tukey's HSD). (C) Representative TLC of crude lipid extracts comparing differences in TAG levels due to hydrogen peroxide application in early stationary phase *P. tricornutum* cultures.

Figure 3: Influence of nitrogen levels on *P. tricornutum* cell density (A), isolated biomass (B) and oxidative stress levels by MDA content (C) combined with hydrogen peroxide application. In A, time = 0 h represents where media from *P. tricornutum* at early stationary phase was removed and replaced with either nitrogen replete (100% N) or deplete (0% N) media. The arrow at time = 48 h represents the time of hydrogen peroxide application. Samples were collected at time = 72 h (24 h after peroxide treatment) to measure biomass (B) and MDA levels (C). Data shown as mean $\pm$ SD (n = 3).

FIGURE CAPTIONS CONTINUED

Figure 4: (A) Influence of nitrogen and phosphate levels on neutral lipid content per biomass in *P. tricornutum* 24 hours after hydrogen peroxide application. Conditions represent three biological replicates each with three technical replicates (n = 9) per analysis and conditions denoted with similar letters did not differ significantly ($p < 0.05$) (ANOVA, Tukey's HSD). (B) Representative TLC of crude lipid extracts comparing changes in TAG levels resulting from the presence or absence of nitrogen and/or phosphate during simultaneous hydrogen peroxide treatment in *P. tricornutum* cultures.

Table 1: Hydrogen peroxide effect on neutral lipid production in early stationary phase *P. tricornutum*.

	<u>Neutral lipid content of isolated biomass (mg/mg)</u>		<u>Total neutral lipids harvested (mg/L)</u>	
3 hours post H₂O₂ application	Mean	% Change	Mean	% Change
H ₂ O	0.19 ± 0.04 ^a	NA	58 ± 14 ^a	NA
0.25 mM H ₂ O ₂	0.19 ± 0.05 ^a	NA	53 ± 13 ^a	NA
0.75 mM H ₂ O ₂	0.21 ± 0.04 ^a	NA	57 ± 14 ^a	NA
2 mM H ₂ O ₂	0.23 ± 0.06 ^a	NA	56 ± 15 ^a	NA
24 hours post H₂O₂ application	Mean	% Change	Mean	% Change
H ₂ O	0.23 ± 0.03 ^a	0%	64 ± 9 ^a	0%
0.25 mM H ₂ O ₂	0.34 ± 0.09 ^b	47%	91 ± 19 ^{b*}	42%
0.75 mM H ₂ O ₂	0.54 ± 0.08 ^c	133%	94 ± 16 ^b	47%
2 mM H ₂ O ₂	0.59 ± 0.08 ^c	156%	96 ± 18 ^b	50%

Data shown are averages ± SD (standard deviation)

Different superscript letters within column indicate significant differences from water-treated controls (ANOVA, Tukey's HSD, $p < 0.05$).

*A statistical outlier was determined in one technical replicate of the dataset for 0.25 mM H₂O₂ total neutral lipids harvested. The average value reported is calculated with this data point (48 mg/L) removed for this condition ($n = 8$). The inclusion or absence of this data point had no effect on altering the significance difference ($p < 0.05$) observed between the total neutral lipids harvested in the 0.25 mM H₂O₂ condition versus the untreated control.

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538 **Figure 1**

Figure 2

Figure 3

Figure 4