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RESEARCH ARTICLE

Induction of reactive oxygen species in marine phytoplankton under crude oil exposure

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Abstract Exposure of phytoplankton to the water-accommodated fraction of crude oil can elicit a number of stress responses, but the mechanisms that drive these responses are unclear. South Louisiana crude oil was selected to investigate its effects on population growth, chlorophyll a (Chl a) content, antioxidative defense, and lipid peroxidation, for the marine diatom, *Ditylum brightwellii*, and the dinoflagellate, *Heterocapsa triquetra*, in laboratory-based microcosm experiments. The transcript levels of several possible stress-responsive genes in *D. brightwellii* were also measured. The microalgae were exposed to crude oil for up to 96 h, and Chl a content, superoxide dismutase (SOD), the glutathione pool (GSH and GSSG), and lipid peroxidation content were analyzed. The cell growth of both phytoplankton species was inhibited with increasing crude oil concentrations. Crude oil exposure did not affect Chl a content significantly in cells. SOD activities showed similar responses in both species, being enhanced at 4- and 8-mg/L crude oil exposure. *Only H. triquetra* demonstrated enhanced activity in GSSG pool and lipid peroxidation at 8-mg/L crude oil exposure, suggesting that phytoplankton species have distinct physiological responses and tolerance levels to crude oil exposure. This study indicated the activation of reactive oxygen species (ROS) in phytoplankton under crude oil exposure; however, the progressive damage in cells is still unknown. Thus, ROS-related damage in nucleic acid, lipids, proteins, and DNA, due to crude oil exposure could be a worthwhile subject of study to better understand crude oil toxicity at the base of the food web.

Keywords Phytoplankton · Crude oil · The deepwater horizon oil spill · Reactive oxygen species

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Introduction

Natural and anthropogenic sources introduce crude oil into aquatic environments, which can lead to chronic and acute contamination for organisms living within these habitats. Toxic effects of crude oil on phytoplankton have been studied extensively and revealed that phytoplankton, which diverge greatly in physiological properties, vary their response and tolerance to oil toxicants Wang et al. 2008; (Liu et al. 2006; Hjorth et al. 2007; Meng et al. 2007; Ozhan and Bargu 2014). Crude oil has been shown to interfere with photosynthetic processes and respiratory mechanisms and affect total primary production of phytoplankton (Miller et al. 1978; Karydis 1979; Bate and Crafford 1985; Harrison et al. 1986; Aksmann and Tukaj 2008; Gonzalez et al. 2009). Lipophilic oil compounds accumulate in the cell membrane and change its structural and functional properties, including the loss of cell permeability, and cause other types of irreversible damage at the cell surface (Sikkema et al. 1995). Furthermore, toxicity studies have demonstrated that hydrocarbons can cause loss of cell mobility (Soto et al. 1975), DNA damage (Bagchi et al. 1998;

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Tang et al. 2002), prevention of nutrient and CO_2 absorption (Koshikawa et al. 2007), inhibition of nucleic acid and protein synthesis (Chen et al. 2008), chloroplast shrinkage, and loss of pigments in phytoplankton (Smith 1968). Many of these processes involve reactive oxygen species (ROS) (Torres et al. 2008; Lushchak 2011). ROS are produced directly by the excitation of O_2 and the subsequent formation of singlet oxygen or by the transfer of one, two, or three electrons to O_2, which results in the formation of superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), or hydroxyl radical ($HO^{\bullet}$) (Baker and Orlandi 1995). It is important to consider that ROS are the natural byproducts of a number of essential metabolic pathways, including photosynthesis and respiration, signaling molecules during cell differentiation, cell cycle progression, and in response to extracellular stimuli.

The proliferation of ROS is mediated by antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), and non-enzymatic antioxidants, such as glutathione, vitamin E, ascorbate, β-carotene, and urate (Foyer et al. 1997; Noctor and Foyer 1998; de Zwart et al., 1999). The enzymes catalyze the removal of ROS by scavenging and reducing them to less reactive molecules such as oxygen or water. Oxidative stress is a physiological stage characterized by a net increase in ROS that results from an insufficient scavenging capacity of the antioxidant defenses (Baker and Orlandi 1995; Mittler 2002). Thus, when the production of ROS exceeds the scavenging capacity of the antioxidant defenses, extensive oxidative damage to membrane lipids (Gutteridge 1995), DNA (Beckman and Ames 1997), and proteins (Berlett and Stadtman 1997) and peroxidation of lipoproteins (Esterbauer and Ramos 1996) takes place.

To date, no study has shown the formation of ROS under crude oil exposure in order to better understand crude oil toxicity mechanisms on phytoplankton. Previous investigations in the literature have revealed the formation of ROS in phytoplankton subject to polycyclic aromatic hydrocarbons (PAHs) such as phenanthrenequinone, anthraquinone (Tukaj and Aksmann 2007), and fluoranthene (Liping and Zheng 2008) as well as the interference of antioxidant defending system operations after anthracene exposure (Aksmann and Tukaj 2008). The interactions between these hydrocarbon compounds with ROS support our hypothesis that crude oil, which contains thousands of different hydrocarbons, causes oxidative stress and damage in phytoplankton.

Antioxidant enzymes represent a primary mechanism to control concentrations of ROS in all organisms. The first ROS formed is the $O_2^{\bullet-}$ and SOD, which acts as a first line of defense in the enzymatic ROS scavenging system by dismutating $O_2^{\bullet-}$ to H_2O_2 (Bowler et al. 1992) to prevent oxidation of biomolecules and production of $HO^{\bullet}$, one of the most reactive oxygen species known to chemistry (Gregory and Fridovich 1973). In addition to this duty, SOD is also the only enzyme capable of processing the dismutation. Therefore, SOD holds a key position within the antioxidant network. Efficient destruction of $O_2^{\bullet-}$ and H_2O_2 requires the action of several antioxidant enzymes acting in synchrony and the reduction of H_2O_2 to O_2 and H_2O is catalyzed by either the CAT or the GPx enzyme system. Glutathione, which is a part of the GPx enzyme system, has several physiological roles, including the detoxification of ROS in chloroplasts, where it acts as an intermediate in the detoxification of free radicals and peroxides (Meister 1988). The reduced form of glutathione, GSH, is a tripeptide that exists interchangeably with its oxidized form, GSSG, in which two glutathione molecules are linked via a disulfide bond. GSH can be oxidized to GSSG by some ROS, such as H_2O_2. In active oxygen elimination, GSH is partly oxidized to GSSG, and the glutathione redox state (GRS)=[GSH/(GSH+0.5 GSSG)] is a useful indicator of oxidative stress (Agrawal 1992; Zenlinski et al. 1999). A failure of the antioxidant defense system to prevent efficient $O_2^{\bullet-}$ and H_2O_2 proliferation may also result in a variety of oxyradical-induced perturbations, including lipid peroxidation. It is also a widely recognized consequence of oxyradical production (Winston and Di Giulio 1991).

It is possible that other cellular processes may respond to disruptions in cellular equilibrium during crude oil exposure. In addition to ROS-induced stress, quantification of gene expression may indicate which biological pathways are affected by contamination in the target organisms. Phytoplankton have been previously used to detect alterations at the transcript level due to environmental stressors such as PAHs (Bopp and Lettieri 2007), light (Schroda et al. 1999), phycotoxin (Yang et al. 2010), and herbicides (Qian et al. 2008).

In the current study, we assessed the cellular and subcellular level responses of two marine phytoplankton species, *Ditylum brightwellii* and *Heterocapsa triquetra*, exposed to elevated concentrations of crude oil above and below their EC_{50} values. The present work was therefore focused to investigate (1) changes in chlorophyll a (Chl a) content (2) the role of ROS and oxidative stress in phytoplankton under crude oil exposure, and (3) the changes at the transcript level of selected genes (Table 1) during crude oil exposure.

Materials and methods

General experimental setup and microalgal cultures

Controlled laboratory microcosm studies were conducted using standard static non-renewal exposure toxicity tests. Control flasks and crude oil-contaminated treatments were set up. All flasks were started with approximately 8×10^3 cell/mL *D. brightwellii* and 12×10^3 cell/mL *H. triquetra* cell concentrations and were inoculated during their exponential growth phase. Each phytoplankton species was exposed to three water-accommodated fractions of the crude oil

Table 1 Details on genes analyzed by quantitative RT-PCR for *Ditylum brightwellii*

Gene	Abbreviation	Direction	Sequence (5 –3)	Cellular process involved	GenBank ref.	Amplicon size (bp)
Heat shock protein 70	Hsp70	F-primer R-primer	CATGTTAGCGGAAGCTGAAAA CAAAACATAAAGTTTCCG CTTGAT	Stress response	AFO84296.1	98
Heat shock protein 90	Hsp90	F-primer R-primer	ACGAGCCAGCCACTTTTTCT TGTCGTCGTCATCCTCATCA	Stress response	AFO84300.1	90
Nitrate transporter	NRT	F-primer R-primer	TGCTGCCATTGCCTCTATTT TCGTCTCGTTGAGCTTGTCA	Transporter	ABP01753.1	86
Photosystem II CP43	psbC	F-primer R-primer	TCCAACTGGTCCAGAAGCAT TGGACCTTGTGCTGATGA AA	Photosynthesis	AGN91125.1	94
Actin	Act	F-primer R-primer	CCCTGGAATCAGTGAGCGTA TGGGGCGACAATCTTAACCT	Cellular structure	AFO84294.1	79

concentrations ranging from 1 to 8 mg/L total petroleum hydrocarbon (TPH). The control flasks contained only the phytoplankton cultures in growth media. Each treatment was conducted in six flasks but every two flasks were pooled together. To ensure that the pooled samples were extracted at similar growth phases, a preliminary study using cultures grown in six different flasks was conducted to determine any significant differences in growth rate. Sampling continued for a total of 4 days with samples taken at 0, 12, 24, 48, 72, and 96 h. At each sampling time point, 60 mL of samples were collected and stored at −20 °C for enzyme analysis. Additional 10–15 mL samples were also taken at each time point for Chl a measurements and cell counts performed under an inverted microscope. The abundance of each phytoplankton species was estimated by enumerating cells on a Sedgwick–Rafter counting slide (n=3).

Autoclaved Pyrex flasks (500 mL) were utilized in all experiments (n=6 for all treatments), and the experimental exposure medium volume was 400 mL for all conditions. Phytoplankton cultures, *D. brightwellii* (CCMP#: 359) and *H. triquetra* (CCMP#: 2981), were provided by the National Center for Culture of Marine Phytoplankton (CCMP), ME, USA. The cultures were grown in f/2 medium (*D. brightwellii*) and f/2-Si medium (*H. triquetra*) at 25 °C and salinity: 35 in 0.22-μm filtered and autoclaved natural seawater. The light source were cool-white fluorescent lights with an irradiance of 85 μE m^{-2} s^{-1} kept on a 12:12-h light/dark cycle.

Preparation of the test media

Recent studies on both fresh and dispersed crude oil toxicity to aquatic organisms have used the water-accommodated fraction (WAF) to provide realistic assessments. We assessed the WAF of two phytoplankton species that are common and abundant in the Gulf of Mexico. Non-weathered Louisiana sweet crude oil (LSC) was collected by British Petroleum (BP) through a riser vent pipe from the damaged wellhead of the Deepwater Horizon drilling rig in the Gulf of Mexico on May 20, 2010, and stored at −4 °C (BP, Ford Collins, CO, USA). The WAF was prepared according to the method described in The Chemical Response to Oil Spills: Ecological Research Forum (CROSERF 2005). The WAF mixtures used in algae toxicology tests were prepared with 0.22 μm filtered and autoclaved Gulf of Mexico seawater in 2 L Klimax valved outlet reservoir bottles. The same seawater used to make WAF mixtures was used to make the growth medium as described above. Loading of 40 g LSC in 1.6-L seawater is known to result in 20–25 % headspace by volume in each bottle. The WAF solutions were prepared at low mixing energy (no vortex). Replication of these conditions involved creating a seawater sample with an oil film on top that is not disturbed by vortex formation. The stirring rate was adjusted to 160 rpm to prevent micro particulate settlement. After 24 h, the samples were settled for 6 h. Samples from the WAF were withdrawn through a valve located at the bottom of the bottle to avoid disturbing the water/oil interface. Samples for chemical analysis were collected in amber glass jars with Teflon-lined caps, allowing no headspace and stored at 4 °C. Serial dilutions (10, 40, and 80 %) of the water phase from each test medium yielded concentrations ranging from 1 to 8-mg/L total petroleum hydrocarbon (TPH) used in the experiments.

Chemical analysis of the crude oil

TPH analysis was carried out with total scanning fluorescence (TSF) (Aqualog, Horiba Scientific), which measured standards and samples at an excitation wavelength of 260 nm and an emission wavelength of 360 nm. Standard solutions were prepared with direct dissolution of LSC in dichloromethane (DCM). The stock solution was diluted to concentrations of 1–20 mg/L. For unknown samples, 100 mL of the WAF was placed in a 250 mL separatory funnel, and 20-mL DCM was added to the first extraction. The aqueous layer was extracted with additional DCM (2×20 mL), and the DCM layers

were combined and dried over Na_2SO_4. The extracts were reduced on a rotary evaporator, yielding a pale yellow liquid. The reduced extracts were transferred to graduated flasks under nitrogen gas and a water bath in a nitrogen evaporator (N-EVAP 111; Organomation Associates, Inc., MA, USA) to reduce them to the desired volumes. A 5-mL sample of the resulting crude was transferred to quartz fluorometer cells (10 mm) for TPH measurements. The samples were diluted to prevent quenching effects.

Chlorophyll a content

Comparison of Chl a contents of crude oil-treated phytoplankton to control groups was performed. Samples (10 mL each) were taken from each flask, filtered through Whatman GF/F filters, and stored at −20 °C until extraction. The filters were then extracted for 24 h in 90 % aqueous acetone at −20 °C, and subsequently analyzed for Chl a using a Turner fluorometer (Parsons et al. 1984). In addition to Chl a content, each phytoplankton species was enumerated on a Sedgwick–Rafter counting slide (n=3). The Chl a content was detected for each cell for the control and crude oil-treated samples.

Analysis and extraction of enzymes

SOD activity was chosen as an enzymatic antioxidant index and the glutathione pool, containing both GSH and GSSG activities, was chosen as a non-enzymatic antioxidant response to assess ROS activity of the phytoplankton exposed to crude oil. The role of lipid peroxidation in crude oil toxicity was also examined as an oxidative injury index by measuring hydroperoxide concentrations. SOD, glutathione, and lipid hydroperoxide enzyme-linked immunosorbent assay (ELISA) kits were purchased from Cayman Chemical (Ann Arbor, MI) to analyze the concentrations within the cells.

Superoxide dismutase

Approximately 2×10^5 the phytoplankton cells were collected by centrifugation. The cell pellets were sonicated in 20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose. Cell extracts were centrifuged at 2000×g for 5 min. Supernatants were removed for the assay. One unit of SOD activity (U) was defined as the enzyme dosage used for exhibiting 50 % dismutation of the superoxide radical.

Glutathione extraction

Approximately 2×10^5 the phytoplankton cells were collected by centrifugation. The cell pellets were sonicated in 2 mL of cold phosphate buffer, pH 7.0, containing 1 mM EDTA and were centrifuged at 10,000×g for 15 min. Supernatants were deprotonated by adding an equal volume of the MPA reagent (dissolve 5 g of metaphosphoric acid in 50-mL water) to each sample and mixing by vortexing. The mixture was incubated at room temperature for 5 min and centrifuged at 2500×g for 3 min. The supernatant was carefully collected without disturbing the precipitate. Fifty microliters of TEAM Reagent (4 M solution of triethanolamine in water) per milliliter of the supernatant was added, and the mixture was vortexed immediately for assaying. This method measured the total glutathione (GSH+GSSG). Quantification of only GSSG was accomplished by first derivatizing GSH with 2-vinylpyridine. The addition of 10 μL of 2-vinylpyridine solution (1 M of 2-vinylpyridine in ethanol) per milliliter of the sample was followed by vortexing and incubation at room temperature for 1 h for GSSG determination. GRS was expressed in sulfur atoms and calculated according to the GSH/(GSH+0.5 GSSG) equation.

Lipid hydroperoxide extraction

Prior to extraction of cells, about 100 mL each of chloroform and methanol was deoxygenated by bubbling nitrogen through the solvents for at least 30 min. The deoxygenated chloroform was stored on ice for extraction of the samples. Approximately 2×10^5 phytoplankton cells were collected by centrifugation. The cell pellets were sonicated in HPLC-grade water. Known volumes of sample were transferred to glass test tubes. An equal volume of Extract R saturated methanol (weighing 100 mg of solid Extract R provided with the assay kit into a test tube, then adding 15-mL methanol) was added to each tube and vortexed. One milliliter of cold chloroform was added to each tube and mixed thoroughly by vortexing before centrifugation at 1500×g for 5 min. The bottom chloroform layer was collected by carefully inserting a Pasteur pipette along the side of the test tube and transferred to a new test tube on ice prior to assaying.

Total RNA isolation and reverse transcription

About 80 mL of three replicates of *D. brightwellii* and *H. triquetra* samples, which were exposed to crude oil for 12 and 24 h at 4 mg/L crude oil concentration, and control groups were collected for RNA extraction. Samples were centrifuged at 15,000 rpm for 10 min to collect cell pellets. The pellets were used for RNA extraction, which was performed using the Qiagen RNeasy® Mini Kit. According to the manufacturer's protocol, this extraction procedure efficiently removes all contaminants, such as crude oil. Concentrations of RNA samples were determined via absorbance at 260 nm (Ab_{260}), and the ratios of Ab_{260} to Ab_{280} were approximately 2, confirming the purity of the samples. Each 2 μg of RNA was treated with RQ1 RNase-free DNase I (Promega) according to the manufacturer's instructions. DNase-treated RNA

was transcribed to cDNA using an oligo(dT) primer by the SuperScript III first-strand synthesis kit (Invitrogen) according to the manufacturer's instructions.

Quantitative PCR

Quantitative PCR was performed with an Applied Biosystems ViiA7 real-time PCR system using SYBR Green detection chemistry (Applied Biosystems) and gene-specific primers. Amplification reactions were performed with 10 μL of Master Mix (Applied Biosystems), 0.5 μM each primer, and 4 μL of 1:10 diluted complementary DNA (cDNA) in a final volume of 20 μL. Samples were loaded in triplicate on 96-well optical reaction plates prior to real-time PCR. The reaction conditions were as follows: 1 min at 95 °C, followed by 40 cycles with 15 s at 95 °C and 1 min at 60 °C. Amplicon melting temperature profiles and no-template controls ensured product specificity. Transcript abundance data were evaluated using Q-Gene (Muller et al., 2002), which takes into account the amplification efficiencies of target and reference genes. Actin was used as an internal control, and the relative expression levels of the genes were computed by the 2^−ddCt method of relative quantification (Livak and Schmittgen 2001).

Statistical analysis

Three replicates, unless otherwise stated, were used in each experiment, and all data were expressed as mean values (95 % confidence interval (CI)). The figure for the end point was run as the mean±SD. Results from different treatments were compared statistically using SigmaStat 12.3 software (Systat Software, Inc., San Jose, CA, USA). ANOVA and *t* test were performed to evaluate significance of individual differences with a probability threshold of 0.05, followed by a post hoc Tukey test.

Results

The measured crude oil concentrations at the beginning of the experiments were 8.03±0.36, 3.93±0.38, and 0.99±0.06 mg/L for 80, 40, and 10 % WAF dilutions, respectively. Crude oil had an inhibitory effect on the growth of both *D. brightwellii* and *H. triquetra* (Fig. 1). The results in cell density obtained over 4 days of experiments demonstrated that both species had similar responses to crude oil exposure at 1- and 4-mg/L crude oil concentrations. At a concentration of 1 mg/L, neither species showed significant differences in cell density compared to the control groups. Also, exposure to 4-mg/L crude oil moderately inhibited growth of both species. However, the response at 8-mg/L crude oil exposure was significantly different between the two species. While 8-mg/L crude oil exposure caused complete growth inhibition on *H. triquetra*, *D. brightwellii* showed only moderate growth inhibition at this concentration (Fig. 1).

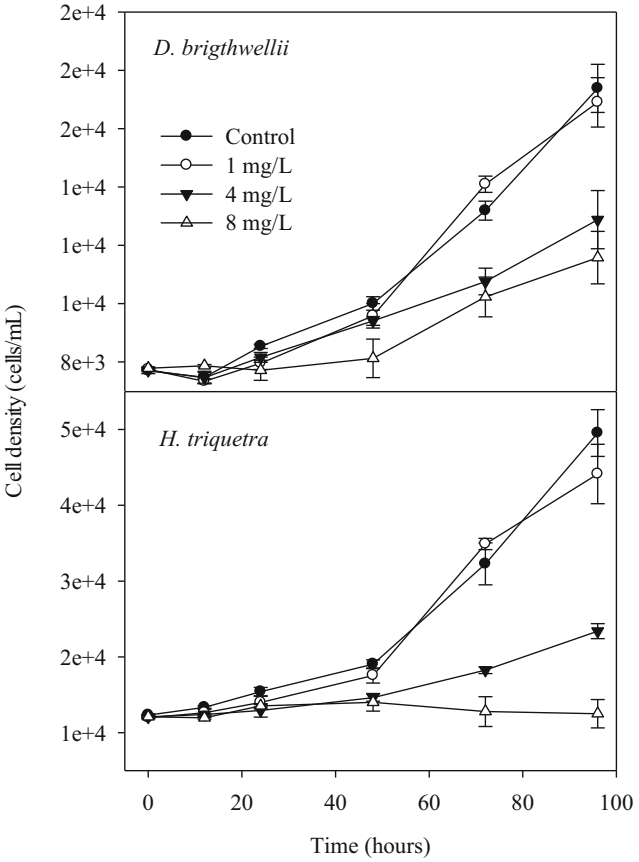

Fig. 1 Growth rate responses of *D. brightwellii* and *H. triquetra* under different crude oil concentrations. Values are the means±SD of three replicates

There was no significant difference in Chl a content between the groups exposed to crude oil and the control groups (ANOVA: $n=6$; $p=0.264$ and $p=0.657$ for *D. brightwellii* and *H. triquetra*, respectively). Although Chl a content was not induced by the addition of crude oil in this study, a number of parameters indicated that cell stress was induced upon addition of the crude oil. SOD activity was analyzed as a parameter of antioxidant response. For both species, SOD activity increased with increasing crude oil concentrations (Fig. 2). However, at 4 mg/L for both species, SOD activities showed a tendency to drop to baseline towards the end of the experiment. At 8 mg/L, SOD activities increased significantly compared to the control groups for both species at 12 h, then slightly dropped and maintained their level throughout the experiment.

Figure 3 shows that total glutathione pools were significantly ($p<0.05$) altered by exposure to 8 mg/L of crude oil in both species. In addition to the highest concentration (8 mg/L) investigated in this study, 4-mg/L crude oil also induced the total glutathione pool significantly ($p<0.05$) in *H. triquetra*.

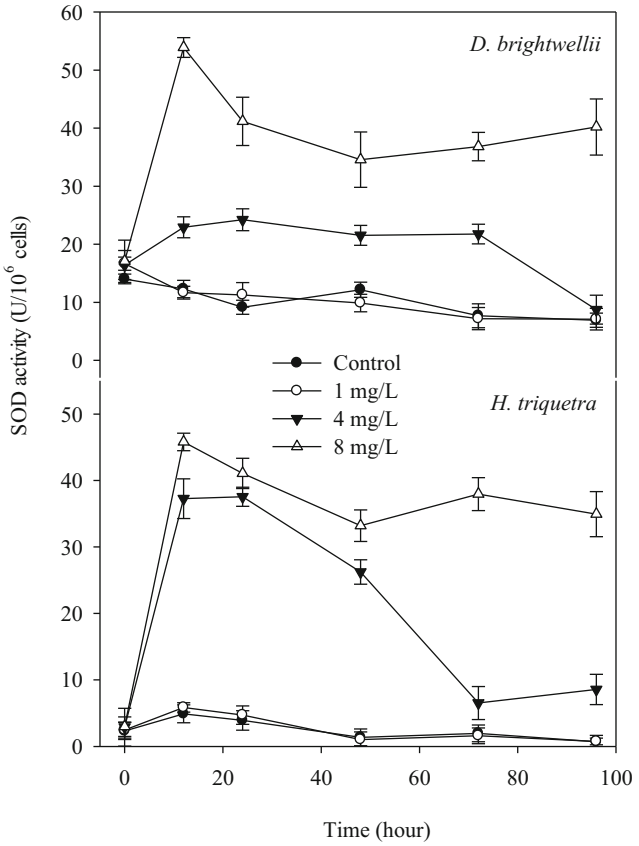

Fig. 2 Superoxide dismutase (SOD) activity in *D. brightwellii* and *H. triquetra* under different concentrations of the crude oil exposure. Data points are averages of replicate samples (n=4)±SD

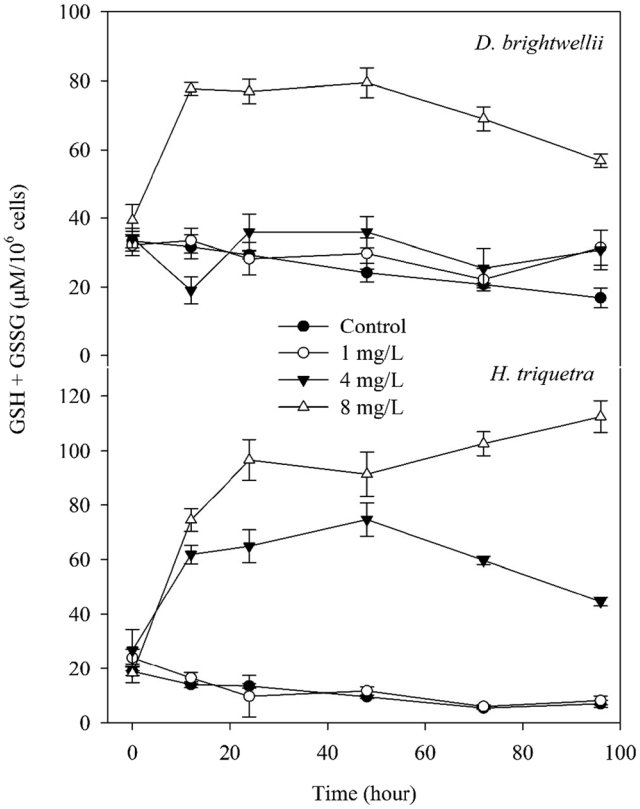

Fig. 3 Total glutathione (GSH+GSSG) activity in *D. brightwellii* and *H. triquetra* under different concentrations of the crude oil exposure. Data points are averages of replicate samples (n=4)±SD

Similar to the total glutathione pool, the GSSG pool was enhanced (p<0.05) by 4- and 8-mg/L crude oil exposure compared to amounts for the control and 1-mg/L treatments for both species (Fig. 4). Overall, *H. triquetra* demonstrated a higher sensitivity to crude oil exposure (p<0.05) in terms of total glutathione and GSSG activity than *D. brightwellii*. Total glutathione and GSSG levels at 8-mg/L for *D. brightwellii* showed a tendency to return to the same level as the control group after 48-h exposure. The increasing SOD activities and total glutathione and GSSG levels indicate that active oxygen production was stimulated by the addition of high crude oil concentration.

Hydroperoxide concentrations in *D. brightwellii* did not change significantly by crude oil exposure; however, exposure of *H. triquetra* to 8-mg/L crude oil led to a significant (p< 0.05) increase in hydroperoxide (Fig. 5). This hydroperoxide stimulation in *H. triquetra* suggests that lipid peroxidation could also be enhanced by crude oil exposure in this phytoplankton.

Observing changes in transcript levels for appropriate genes can indicate early stress responses. Few studies have examined transcript abundance from specific loci in *D. brightwellii* and *H. triquetra*. In addition, only a limited number of gene annotations for putative stress-responsive genes and housekeeping genes in *D. brightwellii* and, in particular, *H. triquetra*, are available in GenBank. To begin to understand the possible gene expression changes associated with crude oil exposure, we used quantitative reverse transcription PCR (qRT-PCR) to measure the transcript abundance of *D. brightwellii* genes encoding heat shock proteins, a photosystem II component, and a nitrate transporter. Comparisons of transcript abundance did not indicate any significantly different up- or downregulation in the genes examined compared to controls under 4-mg/L crude oil exposure (Fig. 6).

Discussion

In this study, growth response of *D. brightwellii* and *H. triquetra* over 4 days of exposure to crude oil varied remarkably at the highest concentration (8 mg/L) of crude oil. Previous studies indicated that crude oil tolerance could vary significantly between different phytoplankton species (e.g., Harrison et al. 1986; Council 2005; Huang et al. 2010; Gonzalez et al. 2013). One study (Ozhan et al. 2014b) clearly showed that at a longer period, the growth responses of these species were highly different when exposed to the crude oil.

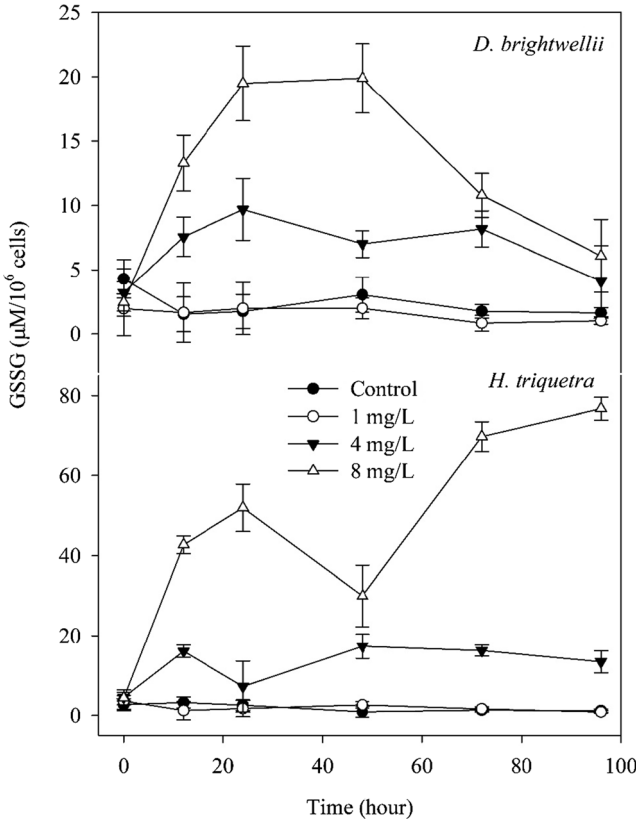

Fig. 4 Oxidized glutathione (GSSG) levels in *D. brightwellii* and *H. triquetra* under different concentrations of the crude oil exposure. Data points are averages of replicate samples (n=4)±SD

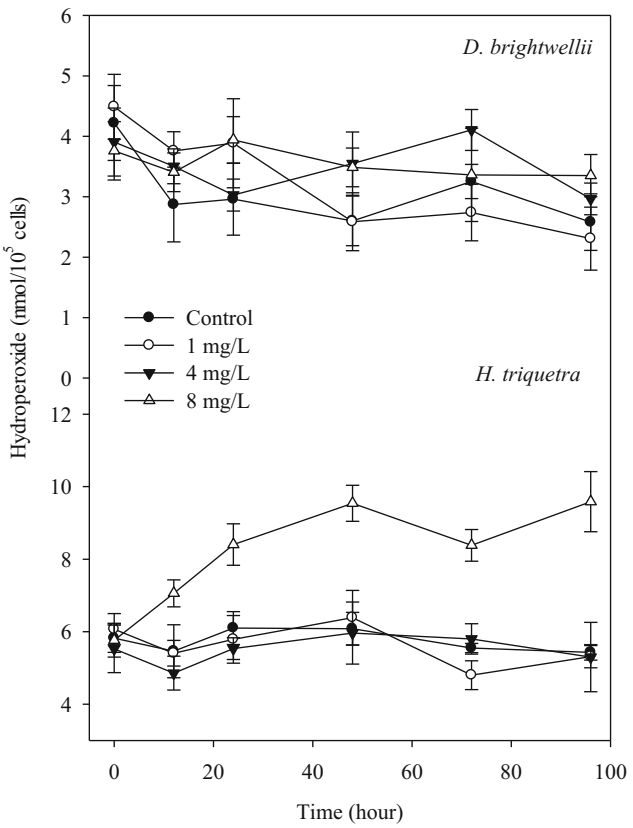

Fig. 5 Hydroperoxide concentration changes in *D. brightwellii* and *H. triquetra* under different concentrations of crude oil exposure. Data points are averages of replicate samples (n=4)±SD

During a 10-day period, while *D. brightwellii* showed high tolerance to the crude oil, *H. triquetra* was dramatically affected.

The increase of crude oil concentration was not accompanied by a change of the Chl a content in cells of *D. brightwellii* and *H. triquetra*. Previously, some studies (Tukaj 1987; Koshikawa et al. 2007; Sargian et al. 2007; Aksmann and Tukaj 2008; Ozhan et al. 2014a) reported changes in chlorophyll pigments as a target for crude oil toxicity in phytoplankton. While Koshikawa et al. (2007) and Aksmann and Tukaj (2008) reported that there was no significant effect of crude oil in Chl a content; Tukaj (1987) and Sargian et al. (2007) observed some changes in Chl a content of phytoplankton under crude oil exposure. Sargian et al. (2007) used ultraviolet-B radiation with crude oil exposure so the measured impact of the two was synergistic. It is difficult to deduce what the sole impact of crude oil to Chl a content may be in this study, since the authors reported that ultraviolet-B radiation alone causes significant changes in Chl a content. In the study by Tukaj (1987), there was no reported quantitative crude oil concentration that could provide a basis for comparison to our conditions. It is possible that the crude oil concentrations used were quite different than those we used in our study.

A very similar response of SOD activities between *D. brightwellii* and *H. triquetra* suggests that these two phytoplankton species were initially impacted by crude oil in a similar way. Activity values for SOD were of the same order of magnitude reported in previous studies (Rijstenbil 2001; Wang and Zheng 2008) despite the use of different xenobiotic stressors. Since this study is the only study that reports the oxidative stress enzyme responses of phytoplankton subject to crude oil exposure, it does not allow for the comparison of enzyme activity levels under crude oil exposure reported in previous literature. Observation of the increased SOD activities only at 12 h suggests that $O_2^{\bullet-}$ production took place at an early stage of the exposure, and then detoxification continued throughout the experiment, particularly for the 4-mg/L crude oil exposure. Since the growth of *D. brightwellii* continued at a reduced rate at 4- and 8-mg/L crude oil exposure, it suggests that resulting oxidative stress by ROS could not reach the threshold value for irreversible damage to the cells. While growth rates for *D. brightwellii* seemed to be similar at 4- and 8-mg/L crude oil exposure, SOD activities showed highly distinctive responses that suggest that *D. brightwellii* could be a bioindicator organism to indicate oxidative stress due to crude oil exposure. The effect on *H. triquetra*'s growth response was clearly discernable after 48 h at 4- and 8-mg/L

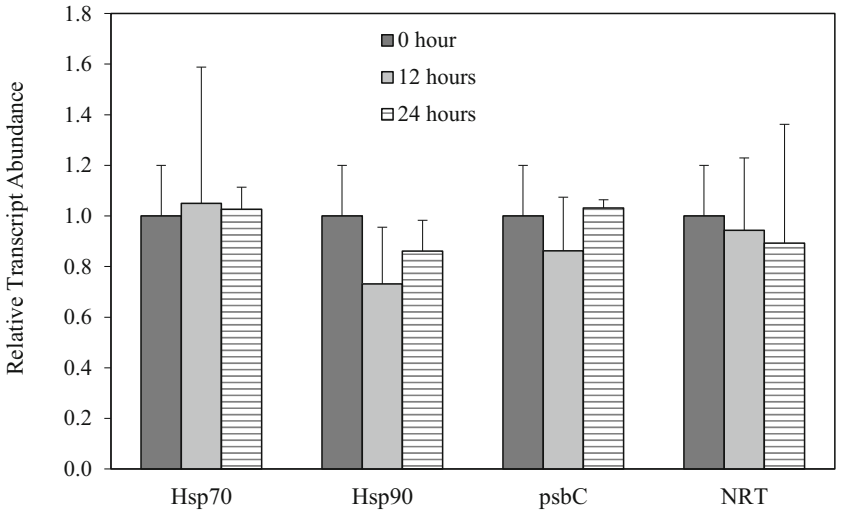

Fig. 6 Transcript abundance for select genes in *D. brightwellii* in response to crude oil. Relative transcript abundance is shown for samples prepared from phytoplankton cultures exposed to 4-mg/L crude oil for 0, 12, or 24 h. The *values* represent transcript abundance of genes normalized to control (0 h) samples. Actin was used as a reference gene. *Error bars* indicate the standard error of duplicate biological samples performed in triplicate (total $n=6$)

crude oil exposure, and SOD activity levels were distinctive after that time point as well. Reduced $O_2^{\bullet-}$ levels after 48 h may have caused the continued growth at 4-mg/L crude oil exposure for this species. Increasing activity of antioxidant enzymes can be expected to reduce oxidative stress to algal cells. However, highly elevated $O_2^{\bullet-}$ levels at 8-mg/L crude oil exposure ceased growth of this species and suggests that the threshold level for irreversible damage for *H. triquetra* was reached.

In both algal species, the activity of SOD, which converts $O_2^{\bullet-}$ into H_2O_2, increased with increasing concentration of crude oil. However, the increase of SOD alone cannot ease the burden of ROS in the cells. Resulting H_2O_2 due to dismutation of $O_2^{\bullet-}$ is a strong oxidant that rapidly oxidizes thiol groups and accumulates in excess within organelles such as chloroplasts, where photosynthesis depends on thiol-regulated enzymes, causing potential harm to cells (Noctor and Foyer 1998). Even though H_2O_2 has more oxidizing power than superoxide, it is biologically less toxic (Miwa et al. 2008). Yet, it must be sequestered by the action of other enzymes such as peroxidases (PODs) and/or CAT enzymes (Cirulis et al. 2013). In addition to these enzymes, GPx, which is one of the major reductants for some of the peroxidase enzymes, takes an active role in detoxification. GPx enzymes catalyze the reduction of H_2O_2 to water and molecular oxygen using cellular glutathione as the reducing agent (Kühn and Borchert 2002). The profile of total glutathione (GSH+GSSG) and GSSG levels showed similarities to SOD activities. Both 4- and 8-mg/L crude oil exposures caused highly significant enhancement of GSH+GSSG and GSSG levels. At this point, it is essential to discuss which part of our glutathione pool data should be used as an indicator of oxidative stress in cells, because the link between glutathione pool alteration and oxidative stress has evolved over time. Previous studies have proposed that rates of glutathione synthesis increase in response to increased H_2O_2 levels (Smith et al. 1984; Smith 1985). But it is well known that in healthy cells, more than 90 % of the total glutathione pool is in its reduced form and that the GSH/GSSG ratio is typically high, greater than 10:1 (Stegeman et al. 1992; Mittler et al. 2004). Under stress conditions, oxidation of GSH to GSSG would decrease GSH levels (Smith et al. 1984) and, subsequently, the level of GSSG increases (Noctor and Foyer 1998). The measurement of elevated GSSG levels, however, suggests that the hepatic GSH/GSSG ratio may be a potential biomarker for oxidative stress (Ballatori et al. 2009; Van der Oost et al. 2003). However, in consideration of excess GSSG in the cell since GR may partially reduce GSSG to GSH during active oxygen production, in more recent years GRS=GSH/(GSH+0.5 GSSG), is therefore commonly used as biochemical measure of oxidative stress (Zenlinski et al. 1999; Rijstenbil 2001, 2002; Schafer and Buettner 2001). At this point, neither GSH+GSSG nor individual GSSG and GSH contents may be used as a proxy for the presence of oxidative stress in cells in this study. To indicate the cells' health, the fraction of GSH in the total glutathione pool was calculated. The results varied between 85 and 95 %, except for *H. triquetra* exposed to 8-mg/L crude oil, which had values that varied between 65 and 84 %. GRS values (Fig. 7) also shows that only a significant reduction was observed for *H. triquetra* at 8-mg/L crude oil exposure. It was the only treatment that showed no growth in this study. In another study, Ozhan et al. (2014b) showed that *H. triquetra*, at 8-mg/L crude oil exposure, showed complete growth inhibition, and all cells died in a 10-day period of crude oil exposure. Thus, GRS values could potentially be used as a stress indicator for cells that were seriously affected by crude oil exposure and had experienced irreversible damage. Unlike *D. brightwellii* (enhanced SOD activity; steady GRS levels), a reduced level of GRS values for *H. triquetra* at 8-mg/L crude oil exposure at the end of the experimental period suggested that *H. triquetra* cells were not able to adapt to the crude oil exposure conditions.

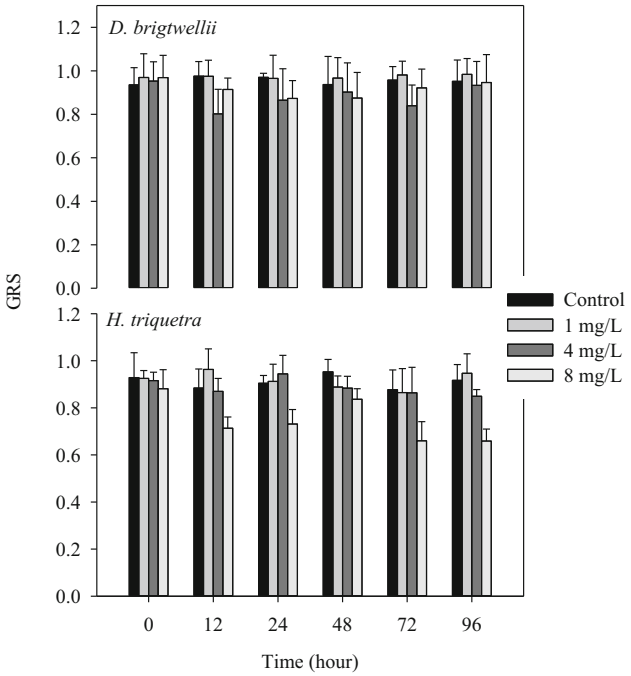

Fig. 7 Glutathione redox state, GRS=GSH/(GSH+0.5 GSSG) values in *D. brightwellii* and *H. triquetra* under different concentrations of crude oil exposure. Data points are averages of replicate samples (n=4)±SD

Lipid peroxidation is often used as an indicator of the effect of ROS-generated oxidative damage (Lushchak 2011). It usually occurs when above-threshold ROS level are reached. Commonly, measuring end products from the degradation of polyunsaturated fatty acid hydroperoxides, such as malonic dialdehyde (MDA) and 4-hydroxy nonenal (4-HNE), are the most widely used assays to monitor lipid peroxidation (Janero et al. 1990; Esterbauer et al. 1991; Pedrajas et al. 1995; Roméo et al. 2003). However, in this study, hydroperoxides were directly measured using the ferric thiocyanate assay (Mihaljevic et al. 1996), which relies on the measurement of ferric ions generated during the reaction of ferrous ion with hydroperoxides. Even though the kit manufacturer claims that ferric ions present in seawater samples are not a source of error, any such errors are easily circumvented by performing the assay in chloroform. The interaction of other trace metals in seawater with these processes is still unknown. The increase in hydroperoxide levels at the 8-mg/L crude oil treatment for *H. triquetra* suggests that antioxidant enzymes induced by the crude oil may not be able to completely eliminate ROS within a short period of time and may cause further damage to pigments, proteins (e.g., Rubisco), nucleic acids, lipid membrane damage, and cell lysis.

Monitoring changes in transcript abundance may allow for the detection of early and/or sensitive stress responses. In the current study, a number of genes were selected as being potentially associated with physiological stress factors. Several genes failed to be reliably detected by the qRT-PCR experiments (data not shown). This could be due to low expression levels in the samples examined or sub-optimal reaction conditions. In addition to two putative housekeeping genes (actin and ubiquitin) the genes that could be accurately tested include those that encode heat shock proteins (Hsp70 and Hsp90), a photosystem II component (psbC), and a nitrate transporter (NRT). The comparison of the relative expression of these genes between crude oil-exposed and control groups provided circumstantial evidence that these genes may not be specifically impacted by crude oil exposure in *D. brightwellii*. Normalization of the transcript levels for the possible stress-responsive genes to either actin (Fig. 6) or ubiquitin (not shown) yielded the same results. Even though heat shock proteins have potential as biomarkers of exposure to environmental contaminants on phytoplankton (Torres et al. 2008), the concentration and duration of crude oil exposure used in this study did not induce heat shock protein genes significantly. The lack of data in the literature about gene expression of phytoplankton under crude oil exposure limits the comparison of our data to any previous study. The closest study was conducted by Bopp and Lettieri (2007), and indicates that PAHs (pyrene, fluoranthene, and benzo [a] pyrene) strongly influenced only select genes involved in fatty acid metabolism and silica shell formation in phytoplankton. Interestingly, the genes involved in photosynthesis were not influenced by PAHs either.

This study provides the first line of evidence that enhanced SOD, glutathione pool activity, and hydroperoxide content are indicative of the ability of biologically active crude oil to promote ROS production, oxidative stress, and lipid peroxidation in phytoplankton. Particularly, distinct responses of these two phytoplankton species in terms of glutathione pool activities and lipid peroxidation suggests that each species has different tolerance levels to crude oil, as confirmed by the different growth responses at high crude oil concentrations. Even though some literature on subcellular response of microalgae to PAH contamination exists (Aksmann and Tukaj 2008; Chen et al. 2008; Wang and Zheng 2008), little attention has been given to subcellular investigations of crude oil toxicity on phytoplankton, making it difficult to link our data to any other damage potentially initiated by ROS, such as damage to nucleic acids, lipids, proteins, and DNA. The exact mechanism of toxicity whereby crude oil inhibited phytoplankton population growth is unknown. Thus, further investigation of damage in cells as the result of oxidative stress is needed to complement the current study.

Conclusions

This study shows that antioxidant enzymes provide protection to phytoplankton species only if crude oil concentration is low enough; in this study, ≤4 mg/L. The data suggests that Chl a content in cells is not affected by crude oil exposure. The

results indicate that high crude oil exposure induces oxidative stress in phytoplankton species. Since SOD is a $O_2^{\bullet-}$ scavenger and glutathione plays an important role in H_2O_2 detoxification, observed changes suggest prolonged and increased levels of ROS production when cells are subject to a 4-day crude oil exposure. The antioxidative defense system was strongly activated, mainly through the activation of SOD. *D. brightwellii* and *H. triquetra* showed distinct responses to crude oil exposure. Evidently, *D. brightwellii* more effectively eliminates excess ROS through its first line of antioxidative defense mechanism than *H. triquetra*, which further serves as a sign of oxidative stress under the crude oil exposure. The gene expression study indicated that though such enzymes activities were triggered at 4-mg/L crude oil exposure, the same concentration of crude oil did not cause significant difference in expression levels of the selected genes between the control group and samples treated with crude oil.

Disclosures The authors have nothing to disclose and report no conflicts of interest.

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