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Growth dynamics and domoic acid production of *Pseudo-nitzschia* sp. in response to oil and dispersant exposure

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ABSTRACT

The diatom genus *Pseudo-nitzschia* is a common component of phytoplankton communities in the Gulf of Mexico and is potentially toxic as some species produce the potent neurotoxin domoic acid. The impact of oil and chemical dispersants on *Pseudo-nitzschia* spp. and domoic acid production have not yet been studied; preliminary findings from a mesocosm experiment suggest this genus may be particularly resilient. A toxicological study was conducted using a colony of *Pseudo-nitzschia* sp. isolated from a station off the coast of Louisiana in the Gulf of Mexico. The cultures were exposed to a water accommodated fraction (WAF) of oil and a diluted chemically enhanced WAF (DCEWAF) which was a mix of oil and dispersant (20:1). Exposure to WAF induced a lag phase but did not inhibit growth rates once in exponential growth. Cultures grown in DCEWAF did not experience a lag phase but had significantly lower growth rates than the Control and WAF cultures. The cellular quota of domoic acid was higher in cultures treated with DCEWAF and WAF relative to their control values, and half of the domoic acid had leaked out of the cells into the surrounding seawater in the DCEWAF cultures while all the domoic acid remained inside the cells in WAF-treated cultures. These results suggest that the presence of oil could lead to toxic blooms, but that the application of dispersant could decrease bioaccumulation of domoic acid through the food web.

1. Introduction

On 20 April, 2010 an explosion at the Deepwater Horizon (DwH) oil rig caused a pipeline to burst on the seafloor, resulting in the continual release of crude oil into the Gulf of Mexico (GOM) at a rate of 50,000–70,000 barrels d^{-1} over a period of almost 90 days (Crone and Tolstoy, 2010; McNutt et al., 2012). Large volumes of the chemical dispersant, Corexit, were applied over large areas of the GOM between 15 May and 12 July to reduce the extent of surface oil slicks (Lehr et al., 2010). Dispersants are a mixture of both anionic surfactants and hydrocarbon-based solvents that lower surface tension at the oil-water interface and allow the formation of smaller oil droplets. While the application of dispersants can increase rates of hydrocarbon biodegradation by marine bacteria (e.g. Passow et al., 2019), they can also introduce more oil into the water column (e.g. Schwehr et al., 2018) and increase the bioavailability of the more toxic fractions, such as polycyclic aromatic hydrocarbons (PAHs), thereby potentially increasing the damage to marine organisms (e.g. Wolfe et al., 2000).

Phytoplankton are a crucial component of marine environments, and diatoms in particular are responsible for ~20% of global primary production (Nelson et al., 1995). The response of diatoms to oil and dispersant exposure is varied, ranging from sensitive (Bretherton et al., 2018; Chao et al., 2012; Deasi et al., 2010; Østgaard et al., 1984; Özhan and Bargu, 2014a) to robust (Bretherton et al., 2018; Garr et al., 2014; Harrison et al., 1986; Hook and Osborn, 2012). In studies on natural phytoplankton assemblages, diatoms typically dominate communities treated with oil (Gilde and Pinckney, 2012; González et al., 2013, 2009; Özhan and Bargu, 2014b) but can be overtaken by other eukaryotes in the presence of dispersed oil (Bretherton et al., 2019; Özhan and Bargu, 2014b). Diatoms are a large component of phytoplankton communities in the GOM, and following the Deepwater Horizon spill their relative abundance increased (Parsons et al., 2015), though the response

Abbreviations: CEWAF, chemically enhanced water accommodated fraction of oil; DCEWAF, a dilute CEWAF; DCM, dichloromethane; EOE, estimated oil equivalents; GOM, Gulf of Mexico; PAH, polycyclic aromatic hydrocarbon; SEM, scanning electron microscopy; WAF, water accommodated fraction of oil

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generally varied on spatial and temporal scales (see Özhan et al., 2014 for a review).

The pennate diatom *Pseudo-nitzschia* is globally distributed genus containing several species that produce domoic acid, a potent neurotoxin (Hasle, 2002). Toxic blooms of *Pseudo-nitzschia* have resulted in the deaths of humans (Bates et al., 1989), sea birds (Fritz et al., 1992), and marine mammals (Fire et al., 2011; Scholin et al., 2000; Silvagni et al., 2005), as well as the closures of commercial fisheries (e.g. Campbell et al., 2003). The abundance of *Pseudo-nitzschia* in the GOM has been increasing since the 1950s due to increased nutrient loading from the Mississippi River (Parsons and Dortch, 2002), and several toxic blooms have been recorded in the area in the last two decades (Dortch et al., 1997; Liefer et al., 2013, 2009; Macintyre et al., 2011). Domoic acid production in toxigenic species is controlled by several abiotic factors including nutrient availability (Bates et al., 1991; Pan et al., 1996b, 1998), irradiance (Pan et al., 1998), and salinity (Thessen et al., 2005). Blooms of *Pseudo-nitzschia* occur in the GOM when N:Si ratios are high since it is a lightly silicifying diatom and can outcompete other species in these conditions (Dortch et al., 1997; Macintyre et al., 2011). The relative abundance of *Pseudo-nitzschia* increased off the coast of Louisiana following the Deepwater Horizon spill (Parsons et al., 2015), and several community-level studies indicate it is robust to oil exposure (Bretherton et al., 2019; Özhan and Bargu, 2014b). However, there is currently limited data on how oil and dispersants influence growth, photosynthesis and domoic acid production in *Pseudo-nitzschia* (Ladd et al., 2018; Özhan and Bargu, 2014b).

In this study, a colony of *Pseudo-nitzschia* recently isolated from the GOM was used to test its response to the presence of non-dispersed and chemically dispersed oil. The water accommodated fraction (WAF) of oil was used as a non-dispersed oil treatment, and a diluted chemically enhanced WAF (DCEWAF) made up of a mixture of oil and Corexit (20:1 ratio) as a chemically dispersed oil treatment. The WAF is a solution of the most water-soluble components (typically those of low molecular weight) released by oil and other petroleum hydrocarbon mixtures when in contact with water (Singer et al., 2001, 2000). Using WAF and DCEWAF ensures that the test organisms are exposed a homogenous hydrophobic oil mixture, and avoids additional problems posed by surface slicks such as limited gas exchange. A range of physiological responses were examined, including changes in growth dynamics, photosynthetic activity, and domoic acid production.

2. Methods

2.1. Cultivation of Pseudo-nitzschia sp

Colonies of *Pseudo-nitzschia* sp. were isolated from the GOM off the coast of Louisiana (28°86 N, 90°49 W) by M. Parsons (Florida Gulf Coast University) on 8 April 2017. Based on observations under the scanning electron and light microscope it is assumed that this is a uni-algal, clonal isolate (genetic characterization was not performed to determine species). The *in-situ* salinity at the isolation site was 32 psu. Prior to experimentation, the colony of *Pseudo-nitzschia* sp. was cultivated in natural seawater collected from the GOM off Galveston, TX and enriched with f/2 nutrients, metals and vitamins (Guillard, 1975). Cultures were monitored daily by counting cells with a haemocytometer, and diluted with fresh f/2 media to maintain a density of ˜100,000 cells mL^{-1} to ensure cells were exponentially growing. The cultures were maintained at a temperature of 19 °C, under a light:dark (L:D) cycle of 12:12 h and a light intensity of 150 μmol m^{-2} s^{-1}.

2.2. Scanning electron microscopy (SEM)

Prior to experimentation, frustules were collected for SEM observations. 10 mL of exponentially growing culture was gently filtered onto a 3 μm polycarbonate filter (Whatman). The filter was rinsed three times with sterile Milli-Q water and placed on a petrislide to dry in a desiccating oven for 24 h. Once dried, a piece of the filter was cut using a scalpel and mounted onto an aluminum stub (Ted Pella) using an adhesive carbon fiber tab (Pelco). The stubs were sputter-coated with a gold-paladium alloy and viewed using a tabletop electron microscope (Hitachi). The transapical length and width of valves, and number of striae and fibulae per 10 μm was used to identify the species according to Tomas (1997).

2.3. Experimental set up

Mixtures of WAF and DCEWAF were prepared using a modified version of the CROSERF method (Singer et al., 2001). Pre-prepared f/2 seawater media was transferred into 1 L glass aspirator bottles with bottom spigots. To each aspirator, 400 μL of either surrogate Macondo Louisiana crude oil (for WAF) or a mixture of the dispersant Corexit and oil in a 20:1 ratio (CEWAF) was added. Each aspirator was then stirred at such a speed that a vortex occupied the upper ˜25% of the volume, and left for 24 h in the dark at room temperature. After mixing, the contents of the 1 L aspirators were pooled into a single 9 L aspirator to form the stock solutions for either WAF or CEWAF. When decanting from the 1 L glass aspirators, the media was passed through a 20 μm nylon mesh sieve to remove large particles and droplets. The surface slick was not allowed to pass through the spigots of the bottles. In order to make the DCEWAF stock solution, a volume of the CEWAF was diluted with fresh f/2 media by a factor of 10.

Stock solutions (850 mL) were transferred into sterile 1 L glass Duran bottles, and inoculated with 150 mL of exponentially growing *Pseudo-nitzschia* sp. culture. Controls were prepared in the same manner, with 850 mL of fresh f/2 media instead. All treatments were prepared in triplicate. Additionally, 500 mL of WAF and DCEWAF stock culture were each transferred to a 1 L Duran bottle, with no phytoplankton added. These formed non-biological controls, and served to correct for background fluorescence from both the oil and dispersant for many of the measurements taken (described below). Sampling occurred at the same time every day (09:30), two hours after the lights came on. The experiment was conducted with batch cultures, and ran until they began entering stationary phase (6 days). Each bottle (triplicates for each treatment) was manually agitated to reduce settling and aerate the media. Algal cultures grown in undiluted CEWAF were also tested, but died off completely after 24 h (data not shown).

2.4. Estimated oil equivalents (EOE)

The oil concentration was monitored daily in each culture vessel by measuring the estimated oil equivalents (EOE; mg L^{-1}). To determine EOE, aliquots (10 mL) were taken from each experimental bottle, as well as initial stock solutions of WAF and DCEWAF, and were extracted into 10 mL dichloromethane (DCM) in 20 mL scintillation vials. DCM allows for detection of oil as low as 0.7 μg L^{-1} (Wade et al., 2013). Approximately 3 mL of the DCM fraction was transferred into a quartz cuvette, and the maximum intensity was measured at an excitation wavelength of 322 nm and an emission wavelength of 376 nm in a Shimadzu spectrofluorophotometer (RF-5301PC, Shimadzu, Houston, TX, USA). A calibration curve was made using dilutions of crude oil in DCM in order to calculate the EOE in each sample. This method is able to measure hydrophilic hydrocarbons, and does not capture fractions such as n-alkanes.

2.5. Oil analysis

The concentration of alkanes and polycyclic aromatic hydrocarbons (PAHs) in the WAF, CEWAF and DCEWAF stocks was determined at the start and at the end of the experiment. 500 mL of each stock was decanted into a glass bottle and immediately stored at -20 °C until analysis. On the final day, samples were collected from each bottle again to measure alkane and PAH concentration.

Hydrocarbon analysis was performed according to a previously established protocol (Bacosa et al., 2016, 2015; Liu et al., 2017) with some modifications (Bacosa et al., 2018). Briefly, the samples were spiked with a mixture of deuterated standards, extracted three times with 20 mL dichloromethane in a separatory funnel, and filtered through a chromatographic column with anhydrous sodium sulfate. The extracts were then concentrated by rotary evaporator and nitrogen gas to 500 µL. Alkanes and PAHs were analyzed using HP-6890 Series GC (Hewlett Packard) interfaced with an Agilent 5973 inert mass selective detector (MSD). The column was an Agilent DB-5MS column. The running conditions are mentioned elsewhere (Bacosa et al., 2018). The hydrocarbons were quantified using the added deuterated standards, and were recovery-corrected.

2.6. Calculations of growth dynamics

A sample for cell counts was taken from each culture bottle every day of the experiment. 1 mL aliquots were preserved in 10% Lugol's solution and stored at 4 °C until processing. The samples were counted with a haemocytometer slide (Neubauer) using a light microscope. Each count for a single replicate bottle was the average of eight counts conducted on a single slide. Growth rates (µ, d^{-1}) were calculated by log transforming the cell counts and fitting a linear regression model using the statistical software package *R* (v 3.5.1).

2.7. Photophysiological measurements

Daily photophysiology measurements were taken using a Fluorescence Induction and Relaxation (FIRe) and Pulse Amplitude Modulation (PAM) fluorometer. Single turnover (ST) induction curves (Kolber et al., 1998) from the FIRe (Satlantic) were used to measure properties such as the quantum yield (F_v/F_m), photosystem II (PSII) antenna size (σ_{PSII}) and PSII turnover time (τ). The PAM (PhytoPAM, Walz) was used to generate rapid light curves (RLCs) in order to calculate maximum relative electron transfer rates (rETRmax). rETRmax is calculated with the following equation:

$$rETRmax = Yield \bullet PAR \bullet 0.84 \bullet 0.5 \quad (1)$$

where rETRmax is the electron transfer rate (µmol electrons m^{-2} s^{-1}), Yield is the F_v/F_m measured at a given light intensity (dimensionless), PAR is the photosynthetically active radiation, i.e. light intensity (µmol photons m^{-2} s^{-1}), 0.84 is the theoretical ratio of photons absorbed by photosynthetic pigments to incident photons, and 0.5 is the theoretical ratio of photons absorbed by PSII relative to photons absorbed by all photosynthetic pigments. For all measurements, samples from the WAF and DCEWAF non-biological controls, as well as filtered seawater, were used to account for background fluorescence.

2.8. Pigment analysis

Samples were taken for analysis of cellular pigment content on the final day of the experiment. 50 mL from each culture was filtered onto a GF filter and stored at -20 °C until processing. To extract pigments, the filter was thawed, placed in a 20 mL scintillation vial with 10 mL of 90% acetone and left in the dark for 2 h at 4 °C. Once the pigments had extracted into the acetone, a 2 mL aliquot was placed into a quartz cuvette for analysis on a spectrophotometer (Shimadzu). Measurements were taken at 630, 644 and 750 nm and both chlorophyll *a* and carotenoid content were calculated after the equations in Ritchie (2006). Non-biological controls for WAF, and DCEWAF were used to correct readings, as well as filtered seawater.

2.9. Quantification of domoic acid

Domoic acid content was measured in all experimental bottles on the final day of the experiment using an enzyme-linked immunosorbent assay (ELISA) kit (Biosense Laboratories). The assay is specific to domoic acid with no cross-reactivity to non-toxic structural analogues such as kainic acid (Garthwaite et al., 2001). The calibrated range of the assay is approximately 10–300 pg mL^{-1}. Two 50 mL samples were taken from each culture, one for total domoic acid and one for extracellular domoic acid. The sample for total domoic acid was sonicated for 5 min and filtered through a 0.8 µm cellulose acetate filter (Thermo Scientific). The resulting filtrate was transferred to a sterile glass bottle. The sample for extracellular domoic acid was gently filtered through a 0.7 µm GF filter (Whatman), and the filtrate transferred to a sterile glass bottle. Samples were stored at −20 °C until analysis.

Domoic acid was extracted from the filtrate by following the protocol included with the ELISA kit. Briefly, filtrate was thawed and diluted with a buffer made of 10% methanol in phosphate buffered saline Tween-20 (PBS-T), termed the Sample Buffer. A 10-point calibration curve was prepared by diluting a domoic acid standard (100 ng mL^{-1}, provided with the kit) with the Sample Buffer in the range of 10,000 – 0.16 pg domoic acid mL^{-1}. A mixture of 1% ovalbumin in Sample Buffer was used for a blank. 50 µL of each of the calibration standard and diluted samples were transferred to a 96-well plastic microplate, where the wells were coated with a domoic acid -conjugated protein. Each well was then also filled with 50 µL of an anti- domoic acid antibody conjugated to horseradish peroxidase (HRP) (anti-domoic acid-HRP), except for the blank. The wells were sealed and the plate was left to incubate for an hour in the dark at room temperature. The free domoic acid in the sample competes with the conjugated domoic acid in the well substrate to bind to the anti-domoic acid-HRP. The higher the concentration of domoic acid in the sample, the fewer antibodies bind to the well.

Once incubation was completed, the plate was unsealed and the contents poured out. Each well was washed four times with 300 µL of PBS-T and then filled with 100 µL of 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate. The plate was left to incubate for 15 min in the dark at room temperature before adding 100 µL of 0.3 M H_2SO_4 to all wells. The TMB peroxidase substrate reacts with the HRP enzyme and gives a blue product. Addition of the acid stops the reaction and changes the color of the product to yellow. The color intensity in the wells was measured spectrophotometrically on a plate reader at 450 nm. Color intensity is higher the more anti-DA-HRP bound to the well substrate and is thus inversely proportional to the domoic acid concentration in the sample. The readings from the plate were converted into ng domoic acid mL^{-1} with a spreadsheet provided by Biosense that used a four-parameter logistic curve fit model to fit the calibration curve. Intracellular domoic acid was calculated by subtracting the extracellular concentration from the total. The data was converted to a cellular toxin quota by dividing the ng domoic acid mL^{-1} by the cell density.

3. Results

3.1. Pseudo-nitzschia identification

The SEM observations showed that the mean transapical width of the frustules was smaller than 3 µm (Fig. 1), which identifies the colony as part of the "*delicatissima* complex" of *Pseudo-nitzschia* (Tomas, 1997). Based on the striae and fibulae density, as well as the origin of isolation, the morphologyof the *Pseudo-nitzschia* colony is consistent with the description of *P. pseudodelicatissima* (Hasle). However, without further genetic or transmission electron microscopy analysis, the species cannot be conclusively identified, and is referred to throughout as *Pseudo-nitzschia* sp.

3.2. Oil concentration and composition

Mean starting estimated oil concentrations in each treatment were 2.58 (± 0.04) mg L^{-1} for DECWAF and 0.25 (± 0.02) mg L^{-1} for WAF

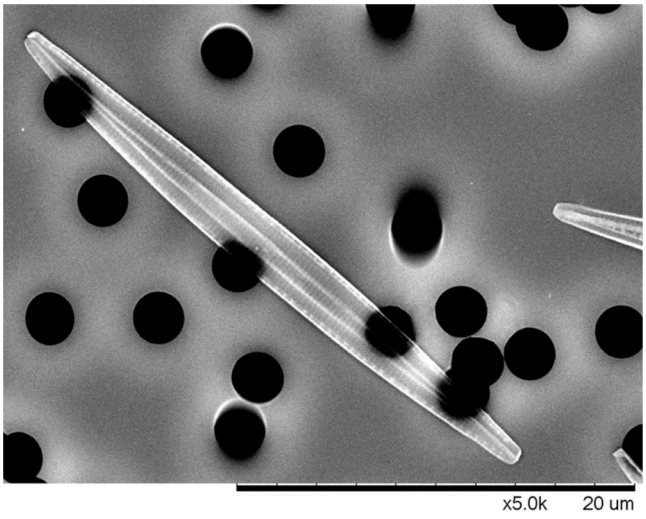

Fig. 1. Scanning electron micrographs of the *Pseudo-nitzschia* sp. colony used in the present study. The transapical width confirms that the colony is part of the *delicatissima* complex.

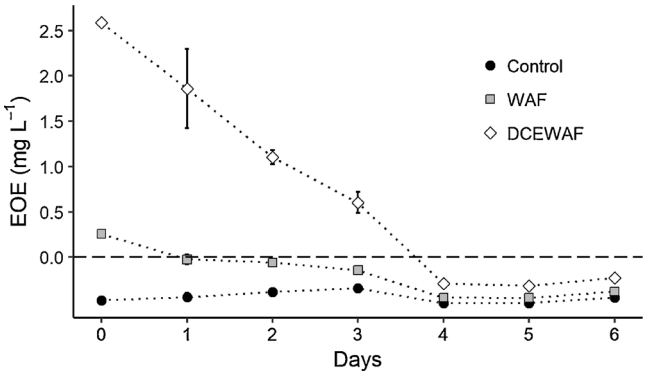

Fig. 2. The estimated oil equivalents (EOE) measured over time in cultures of *Pseudo-nitzschia* sp. grown in oil-only (WAF) and diluted dispersed oil (DCEWAF). Controls had no oil added (f/2 media). Values below the dashed line are below detection limit. Error bars represent standard error, $n = 3$.

Table 1
The concentrations of polycyclic aromatic hydrocarbons (PAH) and alkanes measured in each treatment on day 0 (initial) and day 6 (final) of the experiment.

	Initial PAH (ppb)	Final PAH (ppb)	Initial Alkanes (ppb)	Final Alkanes (ppb)
Control	0.86	0.73	4.38	0.23
WAF	157.18	71.86	154.38	13.01
DCEWAF	37.54	17.95	255.40	24.76

(Fig. 2). In both treatments, EOE declined over time, falling below detection limit in the WAF after 2 days and in the DCEWAF by day 6. The alkane concentration follows a similar pattern to the EOE, with the higher concentrations in the DCEWAF than the WAF (Table 1). In contrast, the concentration of PAHs in the WAF is 4-fold higher than the DCEWAF (Table 1).

3.3. Growth dynamics

Cell densities reached over 200,000 cells mL^{-1} in both the Control (249,166 ± 22,734 cells mL^{-1}) and DCEWAF (232,916 ± 7982 cells mL^{-1}) cultures, and the maximum density reached in the WAF cultures was 171,667 cells mL^{-1} (± 15,143; Fig. 3). The growth rate calculated

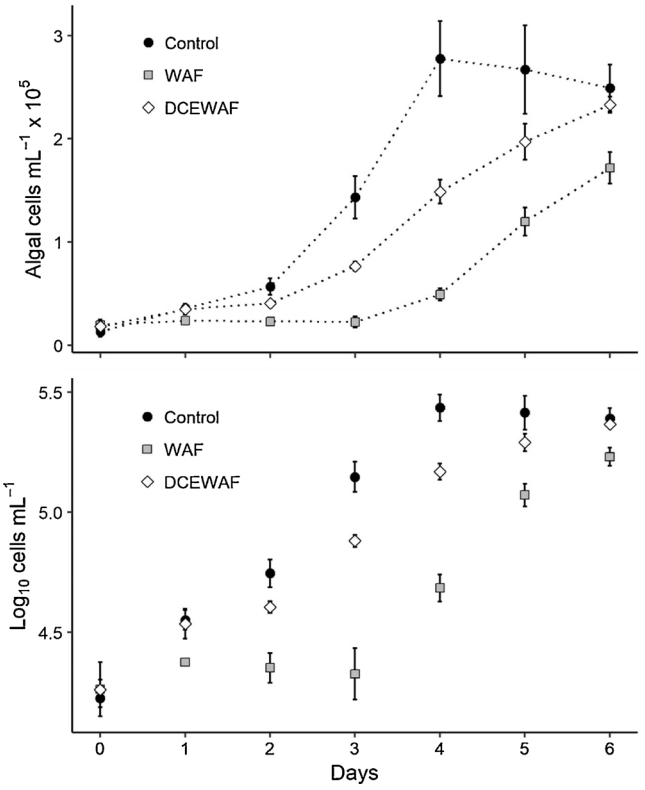

Fig. 3. Cell densities of *Pseudo-nitzschia* sp. cultures grown in oil-only (WAF) and diluted dispersed oil (DCEWAF). Controls were grown in f/2 seawater media. Error bars represent standard error, $n = 3$.

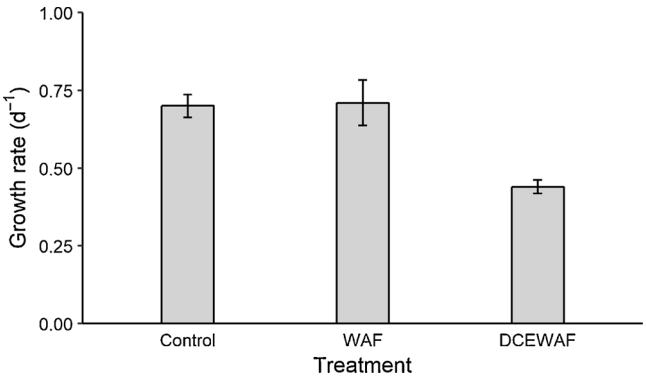

Fig. 4. Growth rates (d^{-1}) calculated for *Pseudo-nitzschia* sp. grown in f/2 seawater media (Control), oil-only (WAF), and diluted dispersed oil (DCEWAF). Error bars represent standard error, $n = 3$.

for the Control and WAF cultures were very similar (0.70 and 0.71 d^{-1} respectively, ± 0.04 and 0.07 respectively), while the growth rate for cultures grown in DCEWAF was significantly lower ($p < 0.05$, one-way ANOVA) at 0.44 d^{-1} (± 0.02; Fig. 4). Neither the Control nor the DCEWAF cultures experienced a lag phase, while the WAF cultures took 3 days to resume exponential growth (Fig. 3). Control cultures reached stationary phase at day 4, while both the WAF and DCEWAF cultures were approaching the end of exponential growth by the end of the experiment (based on cell densities achieved in the Controls). Observations under the light microscope showed that cells exposed to WAF or DCEWAF sometimes became deformed, i.e. bent or crooked (data not shown).

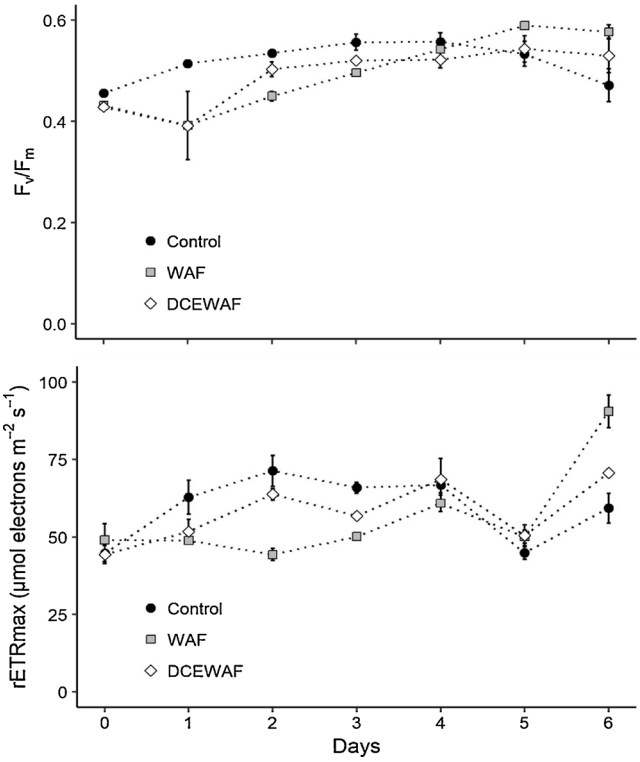

Fig. 5. The photosystem II quantum yield (F_v/F_m) and electron transfer rate (rETRmax) over time in cultures of *Pseudo-nitzschia* sp. exposed to oil-only (WAF) and diluted dispersed oil (DCEWAF). Controls were grown in f/2 seawater media. Error bars represent standard error, $n = 3$.

3.4. Photophysiology and pigment content

The F_v/F_m values in the Control, WAF and DCEWAF cultures was not significantly different ($p < 0.05$, repeated measures ANOVA) throughout the duration of the experiment, and fluctuated around 0.5 for the 6 days (Fig. 5). rETRmax increased initially in both Control and DCEWAF cultures over the first 2 days to ~60 µmol electrons m^{-2} s^{-1}. rETRmax in the WAF cultures remained at ~50 µmol electrons m^{-2} s^{-1} until day 2, after which it began increasing (Fig. 5). Final day rETRmax values were highest in the WAF cultures, followed by DCEWAF and finally Control.

There were no significant changes in any of the other photosynthetic parameters analyzed (Table 2). By the end of the experiment, σ_{PSII} ranged between 285 and 340 quanta $^{-1}$ Å^2 and PSII connectivity ranged between 0.05 and 0.11 in all treatments. The re-oxidation time of the plastoquinone pool (τ1) ranged between 285 and 393 µs, and re-oxidation of PSII (τ2) ranged between 3034 and 4063 µs across all treatments. Chlorophyll *a* content per cell was highest in the Control cultures (12.98 ± 2.46 pg cell^{-1}), followed by the DCEWAF cultures (6.33 ± 0.06 pg cell^{-1}), and the lowest was in the WAF (4.71 ± 0.59 pg cell^{-1}). The carotenoid content followed the same pattern, with highest values measured in the Controls (1.60 ± 0.32 pg cell^{-1}) and the lowest in the WAF (0.61 ± 0.07 pg cell^{-1}).

Table 2
Mean PSII absorption cross section (σ_{PSII}; Å^2 quanta^{-1}), PSII connectivity factor (p; dimensionless), plastoquinone reoxidation time (τ1; µs), PSII reoxidation time (τ2; µs), chlorophyll *a* content (Chla; pg cell^{-1}), and carotenoid content (PCS; pg cell^{-1}) for *Pseudo-nitzschia* sp. grown in Control, WAF, and DCEWAF conditions. Values are means for final day of the experiment, standard errors are in brackets.

	σ_{PSII}	p	τ 1	τ 2	Chla	PCS
Control	339.67 (3.84)	0.067 (0.00)	393.33 (34.35)	4063.00 (586.42)	12.98 (2.46)	1.60 (0.32)
WAF	313.67 (4.06)	0.11 (0.01)	285.00 (10.21)	3034.00 (189.86)	4.71 (0.59)	0.61 (0.075)
DCEWAF	327.00 (13.86)	0.10 (0.01)	330.67 (34.10)	3452.00 (197.65)	6.33 (0.066)	0.85 (0.017)

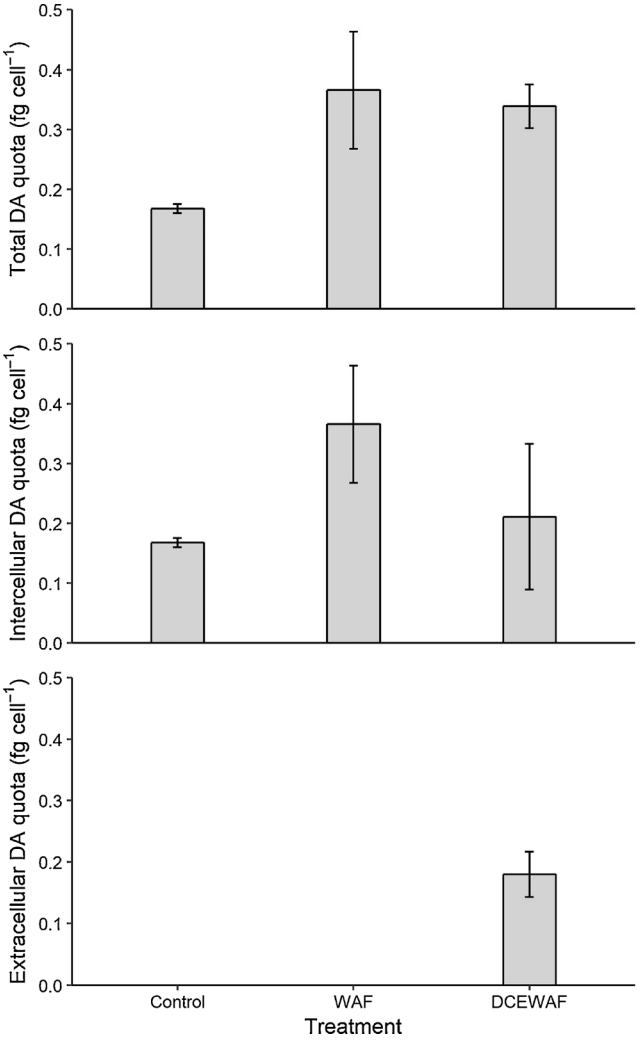

Fig. 6. Cellular quotas of domoic acid (fg DA cell^{-1}) in cultures of *Pseudo-nitzschia* sp. grown in f/2 seawater media (Control), oil-only (WAF), and diluted dispersed oil (DCEWAF). Total and extracellular fractions were measured directly, intracellular was calculated from those two values. Error bars represent standard error, $n = 3$.

3.5. Domoic acid quota

Domoic acid cellular quotas were calculated on day 6 of the experiment (Fig. 6). Both the WAF and DCEWAF quotas were significantly higher ($p < 0.05$, one-way ANOVA) than the Control, with nearly twice the amount detected in the two treatments (0.37 and 0.3 fg cell^{-1} in the WAF and DCEWAF respectively, 0.17 fg cell^{-1} in Control). No extracellular domoic acid was detected in the Control and WAF cultures (Fig. 6). Domoic acid was however partitioned across both intracellular (0.21 fg cell^{-1}) and extracellular (0.18 fg cell^{-1}) fractions in the DCEWAF (Fig. 6). Only the WAF values were significantly higher than the Controls in the intracellular fraction ($p < 0.05$, one-way ANOVA).

4. Discussion

4.1. Impacts to growth dynamics

Exposure to WAF and DCEWAF has been shown to cause longer lag phases and slower growth rates in a range of microalgae, including diatoms (e.g. Bretherton et al., 2018). *Pseudo-nitzschia* has been identified in phytoplankton communities exposed to oil (Özhan and Bargu, 2014b) and dispersed oil treatments (Bretherton et al., 2019). Exposure to oil also caused inhibited growth rates and an increase in cellular domoic acid in cultures of *Pseudo-nitzschia australis* (Ladd et al., 2018), consistent with the findings presented here.

In the present study, the DCEWAF treatment had a higher EOE than the WAF treatment, while the concentration of PAHs in the WAF was much higher (Table 1). This is likely because Corexit preferentially disperses *n*-alkanes (Zhao et al., 2016), which resulted in a greater increase in alkanes than PAHs in the undiluted CEWAF (data not shown). In the present study, exposure to WAF induced a lag phase but did not inhibit growth rates, while DCEWAF did not cause any lag but resulted in slower growth (Figs. 3 and 4). This is also reflected in the differences observed in rETRmax across the cultures. rETRmax increased during the first two days of the experiment in the Control and DCEWAF cultures, but not the WAF cultures (Fig. 5). This would suggest some inhibition of carbon assimilation in the WAF exposed cultures, which they are able to eventually overcome by day 3. Control cultures had reached stationary phase towards the end of the experiment, which is reflected in the decline of rETRmax (Fig. 5).

Work on the model diatom *Thalassiosira pseudonana* suggests that reduced growth could be a result of inhibited silica uptake in the presence of PAHs (Bopp and Lettieri, 2007; Carvalho et al., 2011b; Carvalho and Lettieri, 2011). The process of silicification in diatoms is closely related to the cell cycle, with silica transporting proteins heavily upregulated during S phase (DNA synthesis) (Thamatrakoln and Hildebrand, 2007) and Si-precipitating proteins known as silaffins upregulated during G1 and G2 phase (cell growth) (Frigeri et al., 2006). In the presence of the PAH benzo(a)pyrene *T. pseudonana* has reportedly downregulated production of a silicon transporter enzyme (Carvalho et al., 2011b; Carvalho and Lettieri, 2011), and downregulated the gene *sil3*, responsible for producing a silaffin, in the presence of a variety of PAHs (Bopp and Lettieri, 2007). Batch cultures of *P. multiseries* have shown that onset of stationary phase is often associated with Si depletion rather than nitrogen or phosphorus limitation (Pan et al., 1991). Thus, PAH exposure can stall the cell cycle in interphase by imposing Si-limitation, preventing cytokinesis and potentially causing the lag phase observed in the WAF cultures, where PAH concentrations were highest (Table 1).

Exposure to chemical dispersants and other surfactants can inhibit growth rates in phytoplankton (Poremba et al., 1991; Rial et al., 2013; Sibila et al., 2008). It is thought that dispersant toxicity is brought about by disruption of phospholipid membranes (Hook and Osborn, 2012), and phytoplankton species with thick cell walls or sporopollenin ridges have shown resistance to surfactant damage (Biedlingmaier et al., 1987). In the DCEWAF cultures, exposure to Corexit did not arrest the cell cycle and cause a lag phase, but the ability of the cells to divide was greatly reduced (Figs. 3 and 4). Therefore, while the response in the WAF was possibly driven by PAH concentration, the response of the cultures grown in DCEWAF is more likely a result of the presence of Corexit.

It is interesting to note that in both the WAF and DCEWAF, the PSII quantum yield (F_v/F_m) was not significantly different from the Control (Fig. 5), despite clear inhibition to growth in some way. This is consistent with other studies that also found no changes to photosynthetic performance in the presence of oil or dispersed oil, even when growth was impacted (Bretherton et al., 2018; González et al., 2013, 2009). Other parameters such as PSII antenna size and PSII re-oxidation time (Table 2) are also not significantly different from the Control across the entire experiment. It is clear that this method is not a useful diagnostic tool in detecting phytoplankton that have been exposed to oil and/or dispersants.

4.2. Domoic acid production

There are currently over 35 described species of *Pseudo-nitzschia*, with at least twelve confirmed domoic acid producers (Trainer et al., 2012). The presence of toxigenic species of *Pseudo-nitzschia* have been confirmed in the GOM, with toxin quotas ranging from ~36 fg domoic acid $cell^{-1}$ in isolated cultures (Pan et al., 2001) to 4.5 pg domoic acid $cell^{-1}$ during a bloom off the coast of Alabama (Macintyre et al., 2011). Particulate domoic acid concentrations can become exceptionally high in the coastal waters of Louisiana where this strain was isolated, reaching 13 µg domoic acid L^{-1} (Bargu et al., 2016). Domoic acid was detected in all culture vessels on the final day of the experiment, confirming that the colony was a toxigenic species (Fig. 6). Domoic acid production in *Pseudo-nitzschia* is dependent on growth, with the most toxin produced during late exponential or stationary phase (Bates et al., 1989; Pan et al., 1996b). Typically, lower growth rates result in higher domoic acid production (Pan et al., 1998). Higher cellular domoic acid quotas were measured in the WAF and DCEWAF (Fig. 6), but only the DCEWAF experienced lower growth rates (Fig. 4). This suggests other factors were also influencing domoic acid production.

Nutrient availability also has a regulatory role in the synthesis of domoic acid. For example, P-limited cultures typically produce more domoic acid than those grown in P replete conditions (Fehling et al., 2004; Pan et al., 1996a). Conversely, nitrogen limitation is very unfavorable for domoic acid production, as domoic acid is a nitrogenous compound and nitrogen is required for its synthesis (Bates et al., 1991). Limiting Si can also cause an increase in domoic acid production (Bates et al., 1996, 1989; Pan et al., 1991), which would suggest that PAH exposure could stimulate toxin synthesis by inhibiting Si uptake. At the time of sampling, Control cultures were already in stationary phase, while WAF and DCEWAF cultures had reached late stationary phase (based on cell densities). While the Control cultures were entering nutrient starvation, and therefore more likely to produce domoic acid, the amount of toxin measured was still higher in the WAF and DCEWAF. This suggests that the presence of oil influenced the difference in toxin quota between the Controls and the treatment cultures.

All the domoic acid measured in the Control and WAF cultures was found in the intracellular fraction, but around half of the detected domoic acid in the DCEWAF was measured in the surrounding seawater. Active release of domoic acid has been observed as a response to potentially toxic conditions, such as heavy metal poisoning (Maldonado et al., 2002), however this particular response is largely due to the fact that domoic acid is a strong chelating agent that can bind to metal ions (Maldonado et al., 2002; Rue and Bruland, 2001), rather than a general toxicity response. Since extracellular domoic acid was only detected in the DCEWAF cultures, it is possible that this is a result membrane damage due to the presence of Corexit. While oil exposure caused an overall increase in toxin quota, the addition of dispersant made cells leakier resulting in more domoic acid in the surrounding seawater.

4.3. Implications for toxic blooms in the GOM

Blooms of *Pseudo-nitzschia* become a potential health hazard through bioaccumulation through the food chain (Bates et al., 1989), and domoic acid has been found in the tissues of a number of species that are commercially fished in US waters (see Trainer et al., 2012 for a summary). In the GOM specifically, domoic acid has been found in the tissues of several fish such as Gulf menhaden (Del Rio et al., 2010), striped anchovy, and white mullet (Liefer et al., 2013). Toxic *Pseudo-nitzschia* blooms have also been implicated as the cause of several mass mortality events of bottlenose dolphins throughout the GOM (Fire et al., 2011; Schwacke et al., 2010). If less domoic acid is retained inside the

cell, the amount of toxin transferred through trophic levels is decreased and could potentially reduce the risk posed by a toxic bloom. In other words, the presence of oil pollution might increase domoic acid production per cell, but the application of dispersants could result in a less toxic bloom overall.

There is evidence that *Pseudo-nitzschia* can use domoic acid as a siderophore to scavenge Fe from seawater (Maldonado et al., 2002; Rue and Bruland, 2001). Such siderophores are typically very specific to the organisms that produce them (see Neilands, 1995), and there is some data that suggest that other diatoms are unable to access Fe bound to domoic acid (Prince et al., 2013; Trick et al., 2010). Domoic acid is also released outside the cell in the presence of copepods (Tammilehto et al., 2015), and has been shown to reduce grazing by the copepod *Euphausia pacifica* (Bargu et al., 2006). Free domoic acid also adsorbs to sediments and clay particles (Burns and Ferry, 2007), increasing the amount of toxin found in the particulate phase. Thus, while leakier cells might reduce trophic transfer of domoic acid, an increase in free domoic acid may still have impacts on the marine environment.

5. Conclusions

Exposure to undispersed oil caused a lag period of three days but did not inhibit the cells' ability to divide once in exponential growth. Based on PAH and EOE values, and evidence from the model diatom *T. pseudonana* (Bopp and Lettieri, 2007; Carvalho et al., 2011a), these observations are possibly a result of inhibited silica uptake caused by PAH exposure. Conversely, exposure to a mixture of oil and the dispersant Corexit did not cause a lag period, but significantly reduced the growth rate relative to the Controls. The domoic acid produced by cells in dispersed oil also leaked out of the cell into the surrounding seawater, suggesting that the presence of Corexit damaged the cell membranes. Cell densities in treated cultures still reached numbers similar to those observed during toxic blooms in the GOM (e.g. Liefer et al., 2013), which means that the presence of oil could cause a toxic bloom if *Pseudo-nitzschia* is already present in the area. The addition of dispersant results in more domoic acid released to the surrounding seawater, which could potentially decrease trophic transfer of the toxin, but still impact the marine environment. This study highlights the complex ecotoxicological issues of oil spills and chemical dispersant usage.

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