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The effect of oil sands process-affected water and model naphthenic acids on photosynthesis and growth in *Emiliania huxleyi* and *Chlorella vulgaris.*

Running Title: Effect of naphthenic acids on algae.

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Abstract

Naphthenic acids (NAs) are among the most toxic organic pollutants present in oil sands process waters (OSPW) and enter marine and freshwater environments through natural and anthropogenic sources. We investigated the effects of the acid extractable organic (AEO) fraction of OSPW and individual surrogate NAs, on maximum photosynthetic efficiency of photosystem II (PSII) (F_V/F_M) and cell growth in *Emiliania huxleyi* and *Chlorella vulgaris* as representative marine and freshwater phytoplankton. Whilst F_V/F_M in *E. huxleyi* and *C. vulgaris* was not inhibited by AEO, exposure to two surrogate NAs: (4'-*n*-butylphenyl)-4-butanoic acid (*n*-BPBA) and (4'-*tert*-butylphenyl)-4-butanoic acid (*tert*-BPBA), caused complete inhibition of F_V/F_M in *E. huxleyi* (≥ 10 mg L^{-1} *n*-BPBA; ≥50 mg L^{-1} *tert*-BPBA) but not in *C. vulgaris*. Growth rates and cell abundances in *E. huxleyi* were also reduced when exposed to ≥ 10 mg L^{-1} *n*- and *tert*-BPBA; however, higher concentrations of *n*- and *tert*-BPBA (100 mg L^{-1}) were required to reduce cell growth in *C. vulgaris.* AEO at ≥ 10 mg L^{-1} stimulated *E. huxleyi* growth rate ($p \leq 0.002$), yet had no apparent effect on *C. vulgaris.* In conclusion, *E. huxleyi* was generally more sensitive to NAs than *C. vulgaris*. This report provides a better understanding of the physiological responses of phytoplankton to NAs which will enable improved monitoring of NA pollution in aquatic ecosystems in the future.

1. Introduction

The Athabasca oil sands deposit in Alberta, Canada is one of the largest reservoirs of bitumen in the world, covering an area over 100,000 km^2. Oil sands mining operations currently generate 1.9 million barrels of oil per day and production is expected to increase to 4.8 million barrels by 2030 (CAPP, 2011). Such large-scale industrial operations inevitably have severe environmental impacts. During oil sands mining, large quantities of oil sands process water (OSPW) are generated which are stored in vast tailings ponds. These ponds contribute to the contamination of local aquatic ecosystems (Headley et al., 2004) and pose a threat to environmental and human health (Siddique et al., 2011). The OSPW hydrocarbons comprise mainly asphaltenes, aromatic compounds (typically high molecular weight), alkanes and naphthenic acids (Whitby, 2010; Strausz et al., 2011). Naphthenic acids (NAs) comprise of mixtures of aliphatic, alicyclic and aromatic carboxylic acids, which demonstrate acute and chronic toxicity to several organisms including fish (Young et al., 2007), plants (Kamaluddin and Zwiazek, 2002, Armstrong et al., 2008), bacteria (Frank et al., 2008; Johnson et al., 2011; Johnson et al., 2012) and phytoplankton (Leung et al., 2003). Establishing the environmental impact of NA contamination presents a considerable challenge, since NAs may enter marine and freshwater environments through natural seepages and anthropogenic sources such as discharge from oil refineries and oil spillage events (Brient et al., 1995; Yergeau et al., 2012).

Over the last decade, measurements of chlorophyll fluorescence have become a routine technique for monitoring photosynthetic performance in both higher plants and algae (Baker, 2008). The dark-adapted parameter

F_V/F_M is a measure of the maximum efficiency of photosystem II (PSII) photochemistry. Changes in the value of F_V/F_M provide a simple and rapid way to monitor abiotic and biotic stress in photosynthetic organisms (Baker, 2008, Murchie and Lawson, 2013). Chlorophyll fluorescence measurements (e.g. F_V/F_M) have previously been used in several plant and algal studies investigating the toxicity of heavy metals (Lu et al., 2000) and polycyclic aromatic hydrocarbons (PAHs) (Huang et al., 1997). However, to our knowledge there are no studies that have investigated the effects of OSPW and NAs on F_V/F_M.

In the present study, the marine alga *Emiliania huxleyi* and the freshwater alga *Chlorella vulgaris* were selected as representative phytoplanktonic organisms, since both are biogeographically widespread in their respective environments. The present study aimed to investigate the effects of the acid extractable organic fraction (AEO) of OSPW and individual surrogate NAs, on maximum photosynthetic efficiency of PSII (F_V/F_M) and cell growth in *E. huxleyi* and *C. vulgaris.* Such information is crucial, as it will provide a better understanding of the physiological responses of phytoplankton to OSPW and NAs, thus enabling improved monitoring of NA pollution in aquatic ecosystems.

2. Materials and methods

2.1 *Sources of OSPW and NAs*

Experiments were conducted with surrogate NAs associated with petroleum acids or OSPW and the AEO fraction of OSPW. Two surrogate NAs used in this study were (4′-*n*-butylphenyl)-4-butanoic acid (*n*-BPBA) and

101 (4′-*tert*-butylphenyl)-4-butanoic acid (*tert*-BPBA) and were synthesized using a
102 modified Haworth synthesis (Smith et al., 2008). Both compounds have
103 structural similarities to those NAs previously found in OSPW (Rowland et al.,
104 2011). OSPW was collected at a 2m depth from a Suncor tailings pond
105 (courtesy of L. Gieg, University of Calgary, Canada). The AEO fraction of
106 OSPW was extracted from 1 L OSPW using an ethyl acetate liquid-liquid
107 extraction procedure and the total acid concentration determined by GC-MS
108 as described previously (Johnson et al., 2011). NA and AEO stock solutions
109 were prepared using 0.1 M NaOH to final concentrations of 1, 10, 50 or 100
110 mg L^{-1} (media pH was adjusted to 7.5 for ESAW or 7.1 for BG11 immediately
111 following addition). NA concentrations were selected to include the highest
112 concentration generally observed in OSPW (Holowenko et al., 2002).
113
114 2.2 *Media and growth conditions*
115 Stock cultures of *Emiliania huxleyi* (strain CCMP 370 - a non-coccolith
116 producing strain) and *Chlorella vulgaris* (strain CCAP/211/12) were obtained
117 from the University of Essex culture collection. Both strains were cultured
118 using axenic practices in low light using cool white fluorescent tubes with a
119 light dark cycle of 14:10 at a photon flux density of 150 µmol m^2 s^{-1} in a
120 controlled environment growth room (Fitotron PG660, Sanyo). *E. huxleyi*
121 cultures were grown in 1 L of 0.2 µm filtered ESAW media, pH 7.5 (Berges et
122 al., 2001; Berges et al., 2004), and *C. vulgaris* cultures were grown in 1 L of
123 0.2 µm filtered BG11 freshwater media, pH 7.1 (Berges et al., 2004). Cultures
124 were incubated at 16ºC (within the range for growth of *E. huxleyi*
125 (https://NCMA.bigelow.org) and *C. vulgaris* (Nowack et al., 2005; Shluter et

126 al., 2006), for a total of eight days and harvested for experimental treatments
127 during exponential growth. Triplicate 100 mL sterile serum bottles (Sigma-
128 Aldrich) containing 75 mL filtered media were inoculated simultaneously with
129 either *E. huxleyi* or *C. vulgaris* at an initial cell density of 6 x 10^4 cells mL^{-1}.
130 Cells were acclimated to experimental conditions for 24 h prior to the addition
131 of NAs. Day zero measurements were taken immediately prior to NA addition.
132 Control cultures of *E. huxleyi* and *C. vulgaris* were inoculated into filtered
133 EASW or BG11 media respectively, containing no NAs. Procedural controls
134 containing 75 μL of 0.1 M NaOH (Fisher Scientific) were also established (with
135 media pH adjusted to 7.5 for ESAW or 7.1 for BG11 immediately after
136 addition). Killed controls for all treatments were prepared by heating cultures
137 of *E. huxleyi* and *C. vulgaris* to 60 ºC for 1 h before NA addition and
138 incubation.
139
140 2.3 *Maximum photosynthetic efficiency (F_V/F_M) measurements*
141 Sub-samples (2 mL) were removed daily over the eight day exposure
142 period and dark adapted for 30 min before measuring F_V/F_M, using a Fast^{tracka}
143 II Fast Repetition Rate Fluorometer with a Fast^{act} system (Chelsea
144 Instruments, Molesey, UK).
145
146 2.4 *Cell abundance and light microscopy*
147 Cell density and cell volume measurements were calculated daily using
148 a Z2 Coulter Particle and Size Analyzer (Beckman Coulter, CA, USA). Media
149 blanks were used to account for non-biological particles in the media. Cell
150 fragments were excluded from coulter counter analysis by including a lower

size limit for detection. Growth rates were calculated between days zero and three, during the exponential growth phase of both algae. All cultures were examined by light microscopy on day six using an Olympus BX41 brightfield microscope fitted with a Colorview camera and imaging system (Colorview II).

2.5 *NA extraction and gas chromatography mass spectrometry analysis.*

The cultures that demonstrated significant growth were analysed further for NA degradation as follows: sub-samples (15 mL) were removed at day eight and replicates were pooled together in order to obtain sufficient volume for NA extraction. Killed controls were also extracted for comparison. NAs were extracted using ethyl acetate as described previously (Johnson et al., 2011). Samples were analysed on a 7890A GC system connected to a 5975 VL MS (triple axis detector) and a 120 model autosampler (Agilent Technologies). Samples (1 µl) were injected by splitless injection (270^{o}C injection temperature) onto a 50 m x 320 m x 0.52 µm 19091Z-115E column (Agilent Technologies) using helium as the carrier gas. Oven temperature was set at 50^{o}C for 5 min with an increase to 250^{o}C at a rate of 8^{o}C min^{-1} and a final hold for 15 min. Data was analysed using Chemstation software (Agilent Technologies).

2.6 *Statistical analysis*

Statistical analysis was performed using PASW statistics version 18.0.0. Repeated measures ANOVA was used to determine if significant differences in F_V/F_M occurred throughout the time course of the experiment. If the assumption of sphericity of the data was violated, a Greenhouse-Geisser

176 correction was applied to produce a more conservative F-statistic by reducing
177 the degrees of freedom. Growth parameters and degradation data were
178 analysed using one-way ANOVA with *post hoc* Tukey test.
179
180 **3. Results**
181 3.1 *Effect of the AEO fraction of OSPW and surrogate NAs on maximum*
182 *photosynthetic efficiency (F_V/F_M)*
183 The F_V/F_M of *E. huxleyi* was reduced to zero by day six when incubated
184 with *n*-BPBA at ≥ 10 mg L^{-1} (Fig. 1a). When incubated with *tert*-BPBA, greater
185 concentrations (≥50 mg L^{-1}) were required to cause complete reduction of
186 F_V/F_M in *E. huxleyi* (Fig. 1c). In contrast to the surrogate NAs, the AEO fraction
187 did not inhibit F_V/F_M in *E. huxleyi* and the F_V/F_M remained between 0.39-0.45
188 throughout the eight-day incubation period (Fig. 1e). When *C. vulgaris* cells
189 were incubated with *n*-BPBA, *tert*-BPBA or AEO, no significant differences in
190 F_V/F_M were found in comparison to controls ($F_{13,\ 41}$ = 2.32, p = 0.22). The
191 F_V/F_M parameter remained within the range of 0.43-0.67 for all treatments.
192 This indicates that the surrogate NAs and the AEO fraction of OSPW had no
193 effect on the maximum photosynthetic efficiency of *C. vulgaris* up to 100 mg L^{-1}
194 (Fig. 1b,d and f). The F_V/F_M for all procedural and killed controls also
195 remained constant throughout (Fig. 1), suggesting that any effects observed
196 were not due to the addition of sodium hydroxide.
197
198 3.2 *Effect of AEO fraction of OSPW and surrogate NAs on cell growth*
199 Whilst *E. huxleyi* growth was unaffected by 1 mg L^{-1} *n*- and *tert*-BPBA
200 (Fig. 2, Table 1), greater concentrations (≥ 10 mg L^{-1}) caused significant

201 inhibition of growth. Specifically, growth rates were significantly reduced
202 compared to controls (μ = 0.48) when *E. huxleyi* was exposed to 10 mg L^{-1} *n*-
203 and *tert*-BPBA (μ = 0.07 and 0.31, respectively) ($p \leq 0.001$ in both cases)
204 (Table 1). This resulted in much lower cell abundances at day eight for *E.*
205 *huxleyi* exposed to 10 mg L^{-1} *n*- and *tert*-BPBA (7.94 x 10^3 and 9.01 x 10^5 cells
206 mL^{-1}, respectively) compared to controls (1.97 x 10^6 cells mL^{-1}) (Fig. 2a and c).
207 When *E. huxleyi* was incubated with ≥ 50 mg L^{-1} *n*- and *tert*-BPBA, growth
208 rates were negative (Table 1, Fig. 2a and c).

209 Whilst 1 mg L^{-1} of the AEO fraction of OSPW had no significant impact
210 on *E. huxleyi* growth, greater concentrations (i.e. ≥ 10 mg L^{-1}) resulted in
211 significantly increased growth rates (in the range of μ = 0.64-0.77) compared
212 to controls ($p \leq 0.002$ in all cases) (Table 1). Cell abundances for *E. huxleyi*
213 exposed to ≥ 10 mg L^{-1} of the AEO fraction of OSPW (3.62-5.56 x 10^6 cells
214 mL^{-1}) were also significantly greater at day eight than for controls ($p < 0.001$ in
215 all cases) (Fig. 2 e). The growth of the procedural controls was consistent with
216 the no-NA controls throughout, suggesting that any observed effect was not
217 due to addition of sodium hydroxide (Table 1, Fig. 2a, c and e).

218 Although concentrations of ≤ 50 mg L^{-1} *n*-BPBA had no significant
219 effect on growth rate of *C. vulgaris*, cell densities were significantly reduced
220 (to 9.06 x10^6 cells mL^{-1}) by day eight with 50 mg L^{-1} *n*-BPBA, compared to
221 controls (1.42 x 10^7 cells mL^{-1}) ($p \leq 0.002$). The growth rate for *C. vulgaris*
222 cultures incubated with 100 mg L^{-1} *n*-BPBA was significantly reduced (μ =
223 0.73) compared to controls (μ = 1.15), and cell abundance was almost four-
224 fold lower than controls by day eight (3.60 x 10^6 cells mL^{-1}, $p < 0.001$) (Fig. 2b,
225 Table 2). Whilst the growth rate of *C. vulgaris* did not appear to be

significantly affected by *tert*-BPBA up to 100 mg L^{-1} compared to controls, by day eight, cell densities were significantly lower in the cultures incubated with 50 and 100 mg L^{-1} *tert*-BPBA, (8.95 $x10^6$ and 5.85 $x10^6$ cells mL^{-1} respectively, $p \leq 0.001$ in both cases) (Fig. 2b and d). Exposure to the AEO fraction of OSPW (up to 100 mg L^{-1}) had no significant effect on *C. vulgaris* growth rate or cell density (Fig. 2f, Table 2). Growth from procedural controls was consistent with no-NA controls throughout, suggesting that there was no effect of sodium hydroxide addition (Table 2, Fig. 2b, d and f).

By day eight, *E. huxleyi* cell volumes differed significantly between treatments ($F_{13, 41} = 104.69$, $p < 0.001$) (Table 1). Specifically, cells incubated with 50 mg L^{-1} *n*-BPBA were significantly larger (94.89 μm^3) than controls (82.51 μm^3) ($p = 0.003$) as were cells incubated with 10 and 50 mg L^{-1} *tert*-BPBA (94.06-101.56 μm^3) ($p = 0.003$). In contrast to *n*- and *tert*-BPBA, when *E. huxleyi* cells were incubated with the AEO fraction of OSPW at ≥ 10 mg L^{-1}, cells were significantly reduced in size (40.95-57.35 μm^3, $p < 0.001$ in all cases) compared to controls (Table 1). The cell volume of procedural controls was consistent with no-NA controls at day eight, confirming that there was no effect of sodium hydroxide addition on cell volume (Table 1). *C. vulgaris* cells incubated with ≥ 50 mg L^{-1} *n*-BPBA, and 100 mg L^{-1} *tert*-BPBA had significantly larger cell volumes (between 57.29-79.41 μm^3) compared to controls (50.57 μm^3) ($p < 0.010$ in all cases) (Table 2). The cell volume of *C. vulgaris* cells was not significantly affected by the AEO fraction of OSPW, (up to 100 mg L^{-1}) (Table 2). The cell volume of procedural controls was consistent with no-NA controls at day eight, confirming that there was no effect of NaOH on cell volume (Table 2).

251 3.3. *Effect of the AEO fraction of OSPW and surrogate NAs on cell*
252 *morphology*

253 The effect of the AEO fraction of OSPW and surrogate NAs on cell
254 morphology of *E. huxleyi* and *C. vulgaris* was investigated using light
255 microscopy (Fig. 3). When *E. huxleyi* cells were exposed to 1 mg L^{-1} *n*- or *tert*-
256 BPBA, there was little difference in cell morphology compared to controls (Fig.
257 3c and e). However, when *E. huxleyi* cells were exposed to 10 mg L^{-1} *tert*-
258 BPBA, cells underwent extensive changes in morphology, becoming irregular
259 in appearance. Cell wall damage was apparent and the appearance of several
260 small, round inclusions inside and around cells was noted (Fig. 3d). It was not
261 possible to image cells incubated with ≥ 10 mg L^{-1} *n*-BPBA or ≥ 50 mg L^{-1} *tert*-
262 BPBA due to the toxicity of the NAs resulting in low cell abundances. Image
263 analysis confirmed the observed reduction in the cell size of *E. huxleyi* when
264 exposed to 100 mg L^{-1} AEO fraction of OSPW (Fig. 3f). Microscopy analysis
265 also confirmed the presence of larger *C. vulgaris* cells when incubated with
266 100 mg L^{-1} *n*- and *tert*-BPBA compared to controls, although no dark
267 inclusions were observed in *C. vulgaris* cells incubated with *n*- and *tert*-BPBA
268 as seen in *E. huxleyi* (Fig. 3j and k).
269
270 3.4. *Biodegradation of the AEO fraction of OSPW and surrogate NAs.*
271 Since there were observed differences in NA sensitivity between *E.*
272 *huxleyi* and *C. vulgaris*, it was hypothesised that this was due to differential
273 biodegradation of the BPBA isomers by the two algae. Therefore, the algal
274 cultures that clearly demonstrated growth were further analysed against killed
275 and abiotic controls to determine whether NA biodegradation had occurred

(Supplementary Fig. S1). It was found that whilst *C. vulgaris* cultures partially degraded *n*-BPBA (at 1 and 10 mg L^{-1}) and *tert*-BPBA (at 1 mg L^{-1}), *tert*-BPBA (at 10 mg L^{-1}) and the AEO fraction of OSPW remained. In contrast, *E. huxleyi* cultures almost completely removed *tert*-BPBA (at 1 mg L^{-1}) and partially degraded *tert*-BPBA (at 10 mg L^{-1}) but were unable to degrade either *n*-BPBA (at 1 mg L^{-1}) or the AEO fraction of OSPW (Supplementary Fig. S1). All controls demonstrated no abiotic loss of NAs by photodegradation (data not shown).

4. Discussion

This is the first report to describe the effects of the AEO fraction of OSPW and surrogate NA compounds on maximum photosynthetic efficiency of PSII (F_V/F_M) and cell growth in *Emiliania huxleyi* and *Chlorella vulgaris*. Such information is important as it provides a better understanding of the physiological responses of photosynthetic microorganisms to NAs and may enable improved monitoring of NA pollution in aquatic ecosystems.

Here, we demonstrated that the marine alga *E. huxleyi* was highly sensitive to the surrogate NAs *n*- and *tert*-BPBA at ≥ 10 mg L^{-1}, in terms of photosynthetic efficiency, cell growth and morphology, compared to the freshwater alga *C. vulgaris,* which was more tolerant. Differential sensitivity to the two surrogate BPBA isomers was also observed, whereby *n*-BPBA was generally more toxic than *tert*-BPBA. Similar findings were previously obtained with *n*- and *tert*-butylcyclohexylbutanoic acid isomers using oyster embryos (Smith et al., 2008). In contrast to the results of our study, *tert*-BPBA was previously shown to be more toxic to a bacterial enrichment culture than *n*-

301 BPBA (Johnson et al., 2011). It is well known that NA toxicity can be structure
302 specific, with lower molecular weight acids often demonstrating acute toxicity
303 (Holowenko et al., 2002; Frank et al., 2008). Although the exact mechanism of
304 NA toxicity to algae is unknown, NAs are anionic surfactants (Roberts, 1991)
305 and their acute toxicity is thought to be related to these properties. More
306 specifically, NAs acting as surfactants can disrupt the lipid bilayer of
307 membranes and change membrane properties via polar narcosis (Roberts,
308 1991, Frank et al., 2008). There is also evidence to suggest surfactants
309 interact with and denature cell wall proteins in algae, altering cell permeability
310 and the potential to take in other nutrients and chemicals (Lewis, 1990; Goff,
311 2013).

312 Although differential sensitivity between algal species may be expected
313 (Fairchild et al., 2009), one may hypothesise that the difference observed
314 herein was due to the ability of *C. vulgaris* cultures to more readily biodegrade
315 the BPBA isomers to less toxic metabolites compared to *E. huxleyi*. Indeed, it
316 has been previously shown that biodegradation of the BPBA isomers by a
317 bacterial culture produces ethanoic acid metabolites that are less toxic than
318 the butanoic acid parent compounds (Johnson et al., 2011). In the present
319 study, *C. vulgaris* partially degraded both *n*- and *tert*-BPBA, whilst only *tert*-
320 BPBA was partially degraded by *E. huxleyi*. Previous studies have shown that
321 phytoplankton such as *Selenastrum sp.*, *Navicula sp.* and *Dunaliella* sp. may
322 also degrade certain NAs (Headley et al., 2004, Quesnel et al., 2011). It was
323 also possible that the surrogate NAs were susceptible to photodegradation
324 under UV light, thus reducing their toxicity (Mcmartin et al., 2004; Mishra et
325 al., 2010). However, in the present study, relatively low levels of artificial light

were used with no UV element and abiotic controls showed that photodegradation had not occurred (data not shown).

In contrast to the toxic effects of surrogate NAs observed herein, the AEO fraction of OSPW at concentrations up to 100 mg L^{-1} (i.e. within the top range found in tailings ponds) had no impact on F_V/F_M in either algae species studied. Furthermore, the AEO fraction appeared to have a stimulatory effect on the growth of *E. huxleyi* (but no apparent effect on *C. vulgaris*). Whilst ESAW media is well known to support high growth rates in *E. huxleyi* (Berges et al., 2001; Berges et al., 2004), the apparent stimulation of *E. huxleyi* cells incubated with the AEO fraction of OSPW herein may have been due to the presence of other acid-extractable constituents (Grewer et al., 2010) such as metals and salts, which provided additional nutrients or co-factors for *E. huxleyi*, but not for *C. vulgaris*. NAs have previously been shown to have a stimulatory effect on root and shoot growth in *Arabidopsis thaliana,* which may be due to the broad structural similarity of some NAs to plant growth regulators such as auxins (Leishman et al., 2013). In addition, NAs from OSPW have been shown to stimulate plant growth (as measured by CO_2 uptake) in cattails (*Typha latifolia*) (Wort, 1976; Bendell-Young et al., 2000). Further work is required to determine whether a direct stimulatory effect of the AEO of OSPW occurs in photosynthetic organisms such as the algae studied herein, or whether other, indirect factors such as increased CO_2 uptake also play a role.

In single celled microorganisms it is not uncommon for changes in cell size to occur in response to stress (Li, 1979; Fisher et al., 1981; Goff et al., 2013). In this study, the presence of both *n*- and *tert*-BPBA resulted in an

increased cell size for both *E. huxleyi* and *C. vulgaris,* compared to controls. It is likely that this increase in cell size was in response to toxic stress, whereby a decrease in surface area to volume ratio reduced NA uptake into the cell. Indeed, previous studies have shown that phytoplankton species with a smaller cell size accumulate higher amounts of contaminants such as atrazine (Tang, 1997) and dichlorodiphenyltrichloroethane (Rice and Sitka, 1973) relative to species with a larger cell size, due in part to their larger surface area to volume ratio. Alternatively, increased cell sizes could be due to arrested cell growth cycle prior to cell division or the cells have increased vacuolization, following NA exposure. A similar increase in cell size to that observed in this study has also been noted in other phytoplankton species in the presence of NAs (Goff et al., 2013) and metal contaminants (Li, 1979; Fisher et al., 1981).

In addition to changes in cell size, *E. huxleyi* also underwent changes in morphology following exposure to *tert*-BPBA. Specifically, cells changed from rounded to irregular shape; showed signs of cell wall damage and there was the appearance of several small, round inclusions inside and surrounding cells which may be nuclear fragments resulting from apoptosis. Goff et al. (2013) reported changes to algae morphology following exposure to the NA fraction of OSPW. Specifically, Goff et al. (2013) noted that *Chlamydomonas reinhardtii* cells experienced increased roundness and increased diameter with exposure to NAs. In addition, Goff et al. (2013) described the formation of palmelloids (groups of cells remaining in the remnants of the mother cell wall) when *C. reinhardtii* were exposed to OSPW NAs.

Overall, there was a clear and opposite difference in the sensitivity of the two algae towards surrogate NAs (a toxic response was observed) compared to the AEO fraction of OSPW (a stimulatory response was observed), highlighting a need for caution when extrapolating toxicity data from surrogate NAs, as they may be poor predictors of the response to NAs found in OSPW. The marine alga *E. huxleyi* was highly sensitive to the surrogate NAs, in terms of photosynthetic efficiency, cell growth and morphology, compared to the freshwater alga *C. vulgaris,* which was more tolerant. This report provides a better understanding of the physiological responses of marine and freshwater phytoplankton to surrogate NAs and the AEO fraction of OSPW and will enable improved monitoring of NA pollution in aquatic ecosystems in the future.

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Titles and Legends to Figures

Fig. 1. Effect of acid extractable organic (AEO) fraction of OSPW and surrogate NAs on maximum photosynthetic efficiency (F_V/F_M) in *Emiliania huxleyi* (a, c and e) and *Chlorella vulgaris* (b, d and f) over eight days with (a-b) *n*-BPBA, (c-d) *tert*-BPBA and (e-f) AEO fraction of OSPW at 1 (Δ), 10 (X), 50 (-) and 100 (O) mg L^{-1}, no-NA control (◊) and procedural controls (□). Error bars represent standard deviation of the mean (*n*=3).

Fig. 2. The effect of acid extractable organic (AEO) fraction of OSPW and surrogate NAs on particle (cell) counts of *Emiliania huxleyi* (a, c and e) and *Chlorella vulgaris* (b, d and f). Particle (cell) counts of *E. huxleyi* and *C. vulgaris* over eight days with (a-b) *n*-BPBA, (c-d) *tert*-BPBA and (e-f) the AEO fraction of OSPW at 1 (Δ), 10 (X), 50 (-) and 100 (O) mg L^{-1}. No-NA controls (◊) and procedural controls (□) are also shown. Error bars represent standard deviation of the mean (*n*=3).

Fig. 3. Microscopic analysis of *Emiliana huxleyi* and *Chlorella vulgaris* cells exposed to the acid extractable organic (AEO) fraction of OSPW and surrogate NAs over six days (NAs). Images a-f represent *E. huxleyi* cells incubated with (a) no-NAs, (b) procedural (NaOH) control, (c) 1 mg L^{-1} *tert*-BPBA, (d) 10 mg L^{-1} *tert*-BPBA, (e) 1 mg L^{-1} *n*-BPBA and (f) 100 mg L^{-1} AEO fraction of OSPW. Images g-l represent *C. vulgaris* cells incubated with (g) no-NAs, (h) procedural (NaOH) control, (i) 50 mg L^{-1} *tert*-BPBA, (j) 100 mg L^{-1} *tert*-BPBA, (k) 100 mg L^{-1} *n*-BPBA and (l) 100 mg L^{-1} AEO fraction of OSPW. Scale bars = 10 µm.

Table 1. Growth rates and cell volumes of *Emiliana huxleyi* cultures incubated for eight days with NAs. Values represent means of triplicate samples with standard deviation in parentheses. Growth rates (μ) were calculated over days 0-3. Stars (*) represent results that are statistically different from no-NA controls ($p < 0.05$).

Table 2. Growth rates and cell volumes of *Chlorella vulgaris* cultures incubated for eight days with NAs. Values represent means of triplicate samples with standard deviation in parentheses. Growth rates (μ) were calculated over days 0-3. Stars (*) represent results that are statistically different from no-NA controls ($p < 0.05$).

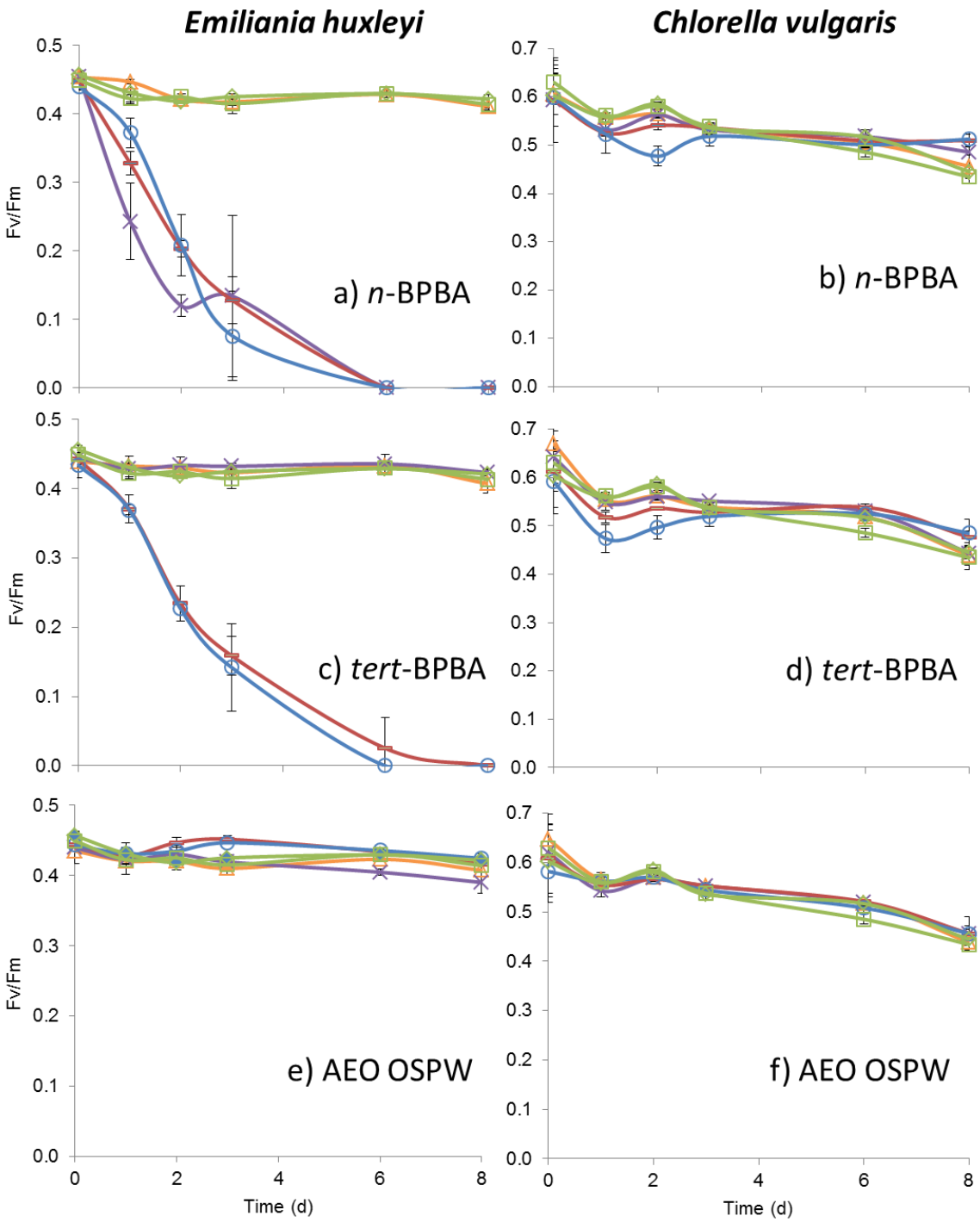
Emiliania huxleyi
Chlorella vulgaris
Fv/Fm
a) n-BPBA
b) n-BPBA
c) tert-BPBA
d) tert-BPBA
e) AEO OSPW
f) AEO OSPW
Time (d)
Time (d)

Fig. 1.

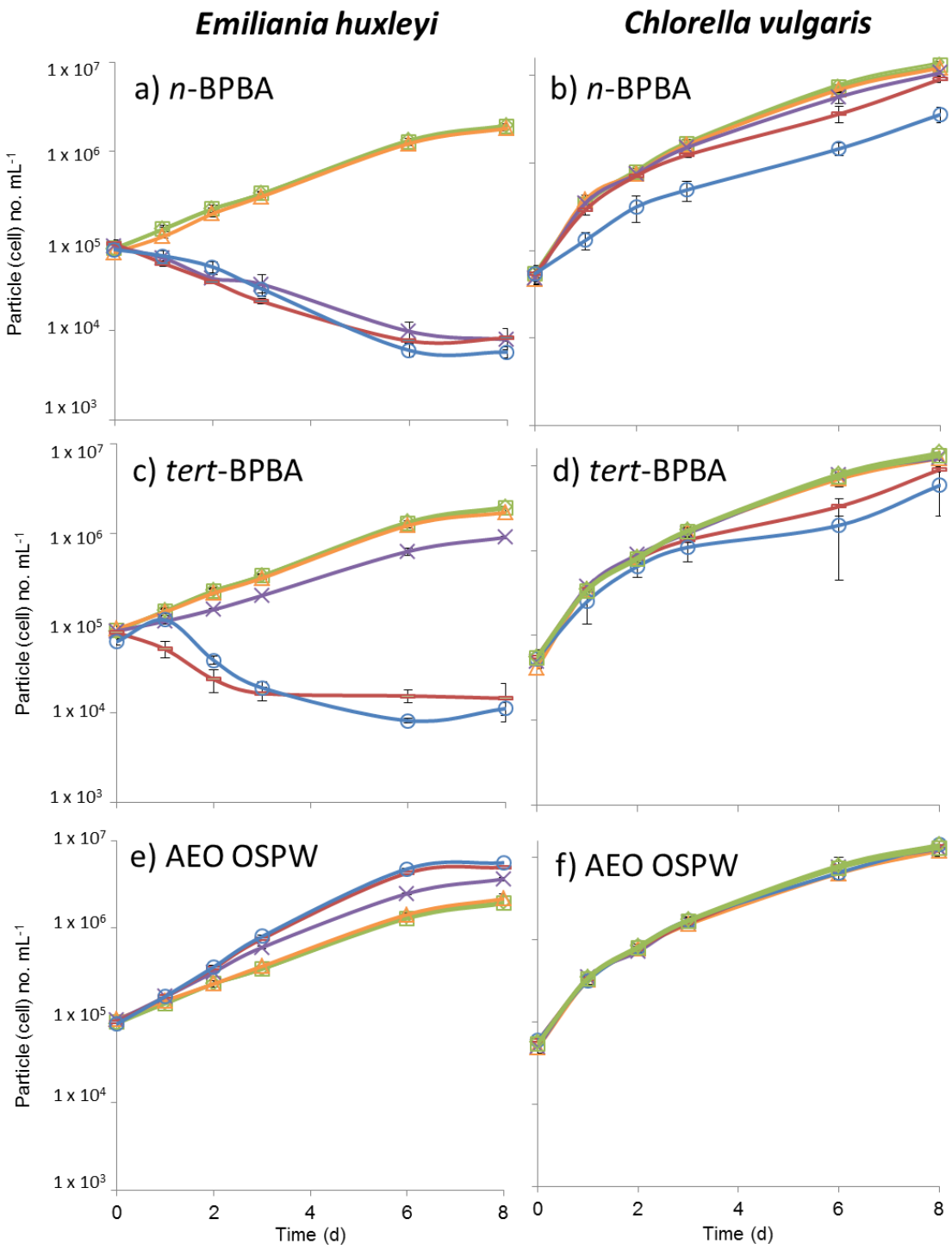
Emiliania huxleyi
Chlorella vulgaris
a) n-BPBA
b) n-BPBA
c) tert-BPBA
d) tert-BPBA
e) AEO OSPW
f) AEO OSPW
Particle (cell) no. mL-1
1 x 10^7
1 x 10^6
1 x 10^5
1 x 10^4
1 x 10^3
0
2
4
6
8
Time (d)

Fig. 2.

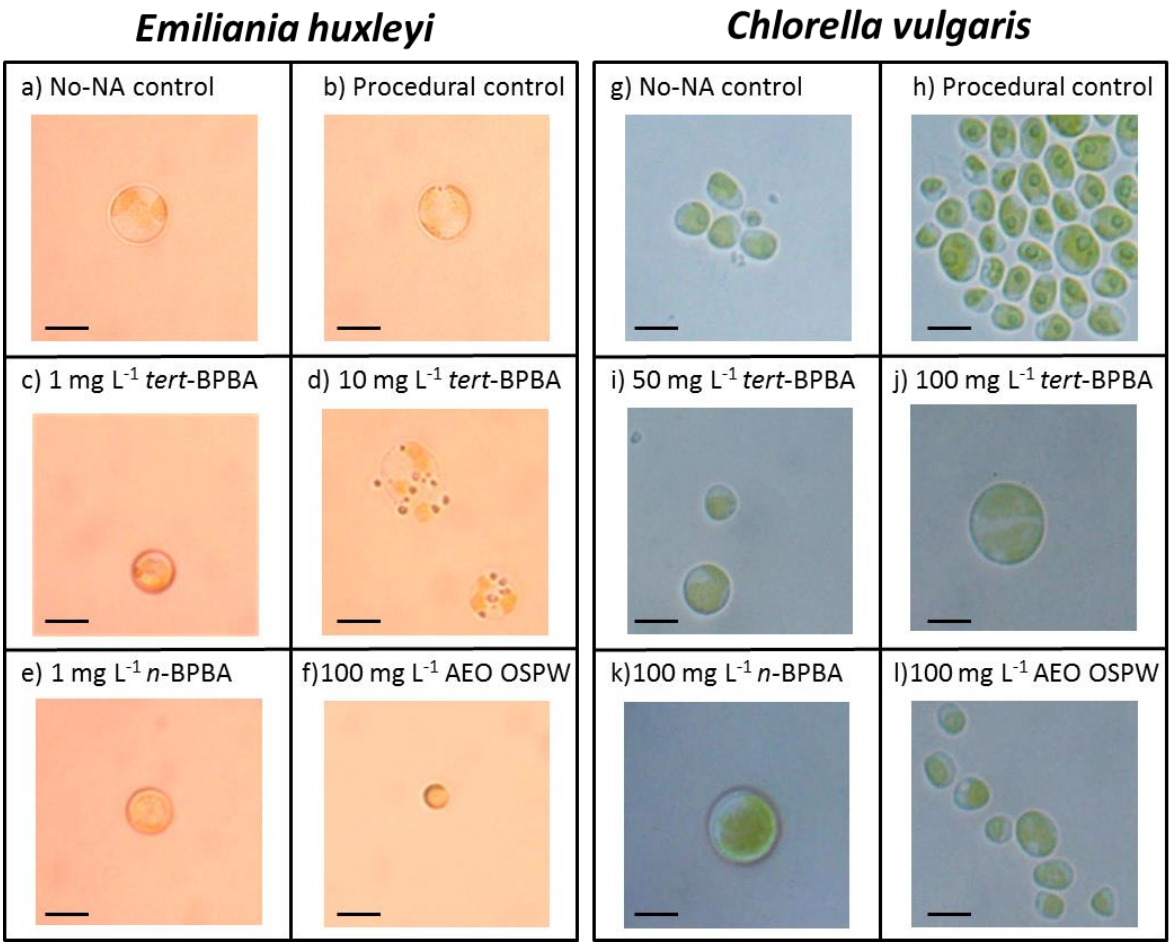
Emiliania huxleyi
Chlorella vulgaris
a) No-NA control
b) Procedural control
g) No-NA control
h) Procedural control
c) 1 mg L-1 tert-BPBA
d) 10 mg L-1 tert-BPBA
i) 50 mg L-1 tert-BPBA
j) 100 mg L-1 tert-BPBA
e) 1 mg L-1 n-BPBA
f)100 mg L-1 AEO OSPW
k)100 mg L-1 n-BPBA
l)100 mg L-1 AEO OSPW

Fig. 3.

652 **Table 1.**

Substrate (mg L^{-1})	Growth rate (μ) calculated over days 0-3	Cell volume Day 0 (μm^3)	Cell volume Day 8 (μm^3)
Control	0.48 (0.01)	70.56 (1.12)	82.51 (4.02)
NaOH control	0.47 (0.02)	70.87 (6.25)	82.52 (3.17)
n-BPBA (1)	0.48 (0.03)	69.29 (5.24)	85.16 (1.57)
n-BPBA (10)	0.07 (0.05)*	71.19 (6.28)	85.32 (4.94)
n-BPBA (50)	-0.49 (0.02)*	73.36 (3.87)	94.89 (0.52)*
n-BPBA (100)	-0.34 (0.02)*	68.20 (2.98)	90.15 (2.19)
tert-BPBA (1)	0.44 (0.02)	67.87 (2.12)	88.06 (6.96)
tert-BPBA (10)	0.31 (0.01)	67.25 (2.12)	101.56 (3.56)*
tert-BPBA (50)	-0.52 (0.10)*	69.14 (2.63)	94.06 (1.70)*
tert-BPBA (100)	-0.40 (0.07)*	73.09 (7.69)	90.29 (0.87)
AEO OSPW (1)	0.46 (0.03)	67.35 (3.40)	76.31 (2.59)
AEO OSPW(10)	0.64 (0.01)*	65.94 (2.14)	57.35 (1.56)*
AEO OSPW (50)	0.73 (0.01)*	71.83 (5.35)	46.08 (2.00)*
AEO OSPW (100)	0.77 (0.02)*	65.36 (5.80)	40.95 (0.75)*

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656 **Table 2.**

Substrate (mg L^{-1})	Growth rate days 0-3 (μ) calculated over days 0-3	Cell volume day 0 (μm^3)	Cell volume day 8 (μm^3)
Control	1.15 (0.06)	72.39 (5.36)	50.57 (0.47)
NaOH control	1.15 (0.09)	68.36 (1.63)	50.86 (2.14)
n-BPBA (1)	1.18 (0.01)	67.12 (0.53)	50.98 (1.80)
n-BPBA (10)	1.15 (0.07)	66.18 (0.83)	51.52 (1.40)
n-BPBA (50)	1.05 (0.08)	67.40 (2.12)	57.29 (2.06)*
n-BPBA (100)	0.73 (0.05)*	64.50 (0.66)	79.41 (2.66)*
tert-BPBA (1)	1.24 (0.03)	62.91 (0.73)	48.47 (0.72)
tert-BPBA (10)	1.16 (0.02)	61.94 (0.97)	45.656 (0.47)
tert-BPBA (50)	1.06 (0.06)	61.70 (1.21)	60.28 (3.80)
tert-BPBA (100)	1.00 (0.11)	60.71 (0.63)	77.42 (17.44)*
AEO of OSPW NAs (1)	1.14 (0.03)	60.09 (0.18)	47.95 (0.43)
AEO of OSPW (10)	1.16 (0.04)	59.92 (0.38)	47.21 (0.63)
AEO of OSPW (50)	1.09 (0.03)	62.47 (0.29)	48.41 (1.05)
AEO of OSPW (100)	1.12 (0.02)	61.15 (0.33)	48.59 (1.01)

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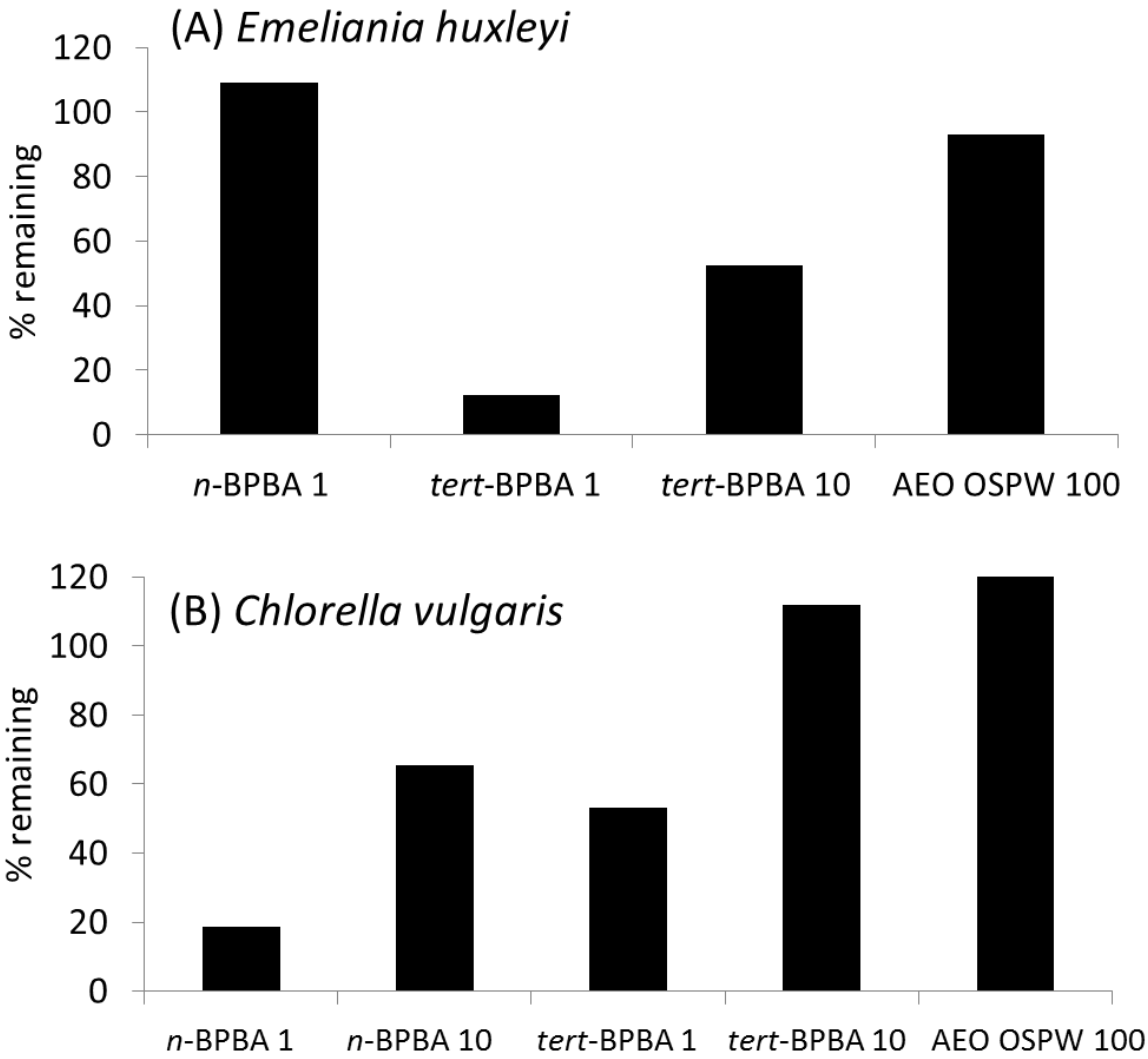
Supplementary Information

Title and Legend to Supplementary Figure

Supplementary Fig. S1. Percentage degradation of individual model naphthenic acids (*n*-BPBA and *tert*-BPBA) and AEO OSPW by (A) *E. huxleyi* and (B) *C. vulgaris*. Bars represent the percentage recovery of pooled triplicate samples following eight days incubation, compared to killed controls. Original NA concentrations (1, 10 and 100 mg L^{-1}) are shown in sample labels (1, 10 and 100 respectively). Values where no algal growth occurred (e.g. with *n*-BPBA and *tert*-BPBA at 100 mg L^{-1}) are not shown.



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671 **Supplementary Fig. S1.**
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The effect of oil sands process-affected water and model naphthenic acids on photosynthesis and growth in *Emiliania huxleyi* and *Chlorella vulgaris.*

Running Title: Effect of naphthenic acids on algae.

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Subject category: Environmental Toxicology and Risk Assessment

Research Highlights:

- *E. huxleyi* was generally more sensitive than *C. vulgaris* to surrogate NAs
- Surrogate NAs at 10-50 mg L^{-1} inhibited F_V/F_M and growth in *E. huxleyi* but not *C. vulgaris*
- F_V/F_M in *C. vulgaris* and *E. huxleyi* was not inhibited by the AEO fraction of OSPW
- The AEO fraction of OSPW at ≥ 10 mg L^{-1} stimulated cell growth in *E. huxleyi*