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Dissolved organic matter release by an axenic culture of *Emiliania huxleyi*

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Measurements of the release of dissolved organic nitrogen (DON) and carbon (DOC) were carried out on an axenic batch culture of the coccolithophorid Emiliania huxleyi. *This unicellular marine alga was cultured using a media with nitrate as the sole N source and the changes of DOM concentrations measured over 14 days. Results showed that there was a significant release of DON, i.e.7.6 μM N day^{-1} during mid-exponential growth phase (days 5–7). The highest release of DOC was also recorded in the same growth phase and accounted for 24.0 μM C day^{-1}.*

Keywords: dissolved organic matter, anexic culture, *Emiliana huxleyi*

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INTRODUCTION

Dissolved organic matter (DOM) cycling has been studied in marine waters worldwide with higher concentrations of DOM generally measured during phytoplankton bloom periods (Kirchman *et al.*, 1991; Gobler & Sanudo-Wilhelmy, 2003; Suratman, 2007). DOM is usually measured by assessing one of its constituent parts, i.e. DOC, DON or dissolved organic phosphorous (DOP). Its production is important due to its potential to drive primary production in marine systems when inorganic nutrients, typically nitrate, are depleted (Carpenter *et al.*, 1972; Berman & Chava, 1999; Suratman, 2007). However, it is unclear whether DOM from phytoplankton is released from healthy cells during phytoplankton growth, or only from dead and decaying phytoplankton (Fogg, 1977; Sharp, 1977; Collos *et al.*, 1992). Collos *et al.* (1992) found extensive excretion of DON equal to 63% of nitrate uptake during the light period for a culture in exponential growth phase. In contrast, it has also been shown that dead and decaying phytoplankton is a major source of DOM in seawater as a result of autolysis or mechanical breakage by grazing zooplankton (Lampert, 1978; Hygum *et al.*, 1997).

The aim of this experiment was therefore to determine if DON and DOC release occurs from healthy cells and also to quantify any such release. The absence of bacteria from the cultures also allowed gross release to be measured since there was no bacterial uptake. To the best of our knowledge, this is the first study measuring DON and DOC simultaneously for a phytoplankton culture, as previous studies have focused on either DON or DOC alone (Newell *et al.*, 1972; Myklestad *et al.*, 1989; Collos *et al.*, 1992; Pujo-Pay *et al.*, 1997; Engel *et al.*, 2004; Mulholland *et al.*, 2004). It is important to monitor the simultaneous release of DOM since this allows the determination of the stoichiometry of nutrient release, and thus has implications for biogeochemical cycling more broadly (Arrigo, 2005; Painter *et al.*, 2007). *Emiliana huxleyi* was selected because it is widespread throughout the world's oceans forming large shelf sea and open ocean blooms (Winter *et al.*, 1994), but is underrepresented in culture studies of DOM.

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MATERIALS AND METHODS

The experiment was carried out with axenic cultures of the warm water strain CCMP 373 of *E. huxleyi*. Details of the media and conditions used can be found in Chance *et al.* (2007). In brief, an experimental stock culture of *E. huxleyi* was grown in f/20 culture media (Guillard, 1975) prepared using seawater from the open Atlantic Ocean. The seawater was filtered through a 0.2 μm cellulose acetate filter (Sartorius USA) and autoclaved prior to addition of sterile nutrient stock solutions. Nitrate was the sole N source, with an initial concentration of ~100 μM in all flasks. The stock culture was kept under 14:10 hours light–dark cycle, at a temperature of 15°C, with light intensities of 40–50 μmol photon m^{-2} s^{-1}. Flasks containing 1 l of growth medium were inoculated with a fixed volume (~50 ml) of stock culture in the exponential growth phase. Control flasks were prepared without the inoculation step. DON and DOC concentrations were determined at t = 0, 5, 7 and 14 days. For DON and DOC determinations, aliquots of 15 ml were taken and filtered through a 0.2 μm filter. Since filtration can potentially cause cell lysis and therefore DOM release, in order to reduce the filtration artefacts (Sharp, 1977; Vogel & Frisch, 1978), filtration was carried out using a syringe filter under gentle, hand-applied pressure. Samples were then analysed by high temperature catalytic oxidation (HTCO) with a Thermalox (UK) TOC/TN analyser (temperature: 680 ± 10°C; catalyst: 0.5% Pt/Al_2O_3) coupled to

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chemiluminescence and non-dispersive infrared (NDIR) detectors for NO_x and CO_2 detection, produced from total dissolved N (TDN) and DOC compounds respectively (Hansell *et al.*, 1993). The instrument used ≥ 3 injections per analysis until a precision of $\pm 5\%$ or better was achieved.

To determine TDN, calibration of the instrument was performed with potassium nitrate in ultrapure water (≤ 18.2 MΩ). The TDN system blank was estimated for each individual run by injected ultrapure water with typical values of 1.6–3.7 μM (2.3 ± 0.6 μM, N = 10) and blank corrections were applied to the TDN data. For DOC analysis, samples were acidified by adding 20 μl of 10% HCl to a 2 ml sample and sparged with high purity O_2 to eliminate inorganic C. Calibration of the instrument was performed by running freshly prepared standards of potassium phthalate in ultrapure water. As for TDN determination, the DOC system blank was also estimated by injected ultrapure water with a typical value 46–57 μM (mean 51 ± 4 μM, N = 10) and blank corrections applied to the DOC data. Certified reference material (CRM) of deep seawater from the Sargasso Sea obtained from the Hansell laboratory (University of Miami, USA) was used during the routine analyses of water samples as recommended by Sharp (2002). The recovery of the TDN and DOC in the CRM was between 91–110% ($101 \pm 6\%$, N = 10) and 99–115% ($110 \pm 6\%$, N = 10) respectively. The precision of analysis for TDN and DOC was $<5\%$. Nitrate + nitrite (hereafter nitrate) was analysed using a Scalar (The Netherlands) San Plus Autoanalyser according to the method of Kirkwood (1996) with an analytical error $<5\%$ relative to Ocean Scientific International (UK) standards. Ammonium was measured using a Jasco fluorometer (UK) according to the method of Holmes *et al.* (1999) with a precision of analysis $<5\%$. DON was calculated as the difference between TDN and dissolved inorganic N (DIN), i.e. nitrate and ammonium.

Chlorophyll-*a* (chl-*a*) and cell counts were measured on each day from t = 0 to 14 days. Chl-*a* was determined by filtering 15 ml of culture through a Whatman (UK) GF/F glass fibre filter, followed by acetone extraction and measurement of fluorescence using a Turner (USA) fluorometer according to the method of Parsons *et al.* (1984). In addition, cell counts were made using a Beckman (USA) Coulter Multi-sizer III electronic particle counter. DAPI staining combined with epifluorescence microscopy (Sherr *et al.*, 1993) was used to confirm that bacteria were absent from all the experimental cultures and the control on day 7 of the experiment.

RESULTS AND DISCUSSION

The growth cycle of *E. huxleyi* was followed using 2 indices of biomass: cell counts and chl-*a* concentrations (Figure 1a, b). No algal or bacterial growth was observed in the control flask. There was a steady decrease of DON concentrations for the control sample (Figure 1c) suggesting depletion of DON due to abiotic processes such as adsorption onto bottle walls (Slawyk & Raimbault, 1995). The DOC concentrations in the control culture remained constant (259 ± 6 μM) throughout the experiment (Figure 1d).

Nitrate concentrations decreased steadily throughout the culture period, falling below the detection limit (<0.1 μM) on day 14. The decreasing nitrate corresponded to an increase

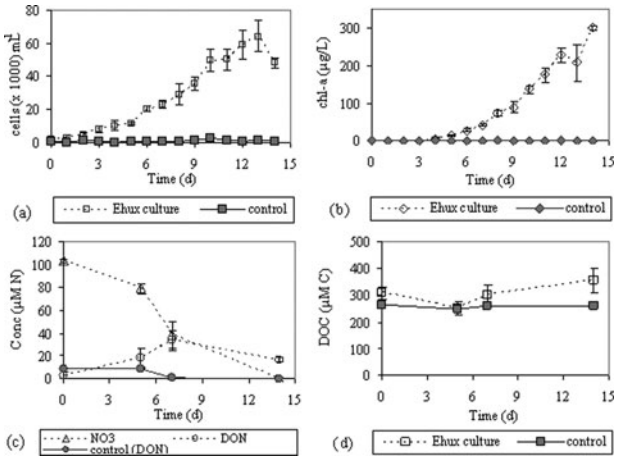

Fig. 1. Changes of (a) mean cell counts, (b) chl-*a*, (c) nitrate and DON concentrations and (d) DOC concentrations with time for *Emiliana huxleyi* (as Ehux in the figure) culture growth in nitrate media. Error bars represent the standard deviations from the means of four replicate cultures for TDN and DOC.

of the algal biomass, as indicated by the increasing cell counts and chl-*a* concentrations. This is due to the conversion of nitrate to particulate organic matter. The DON variations showed a significant difference (ANOVA test, $P < 0.05$) compared to the control with an increase of DON mean concentrations from 3.1 μM to 33.5 μM before decreasing to 16.8 μM at the end of the experiments. Similar to DON, there was a significant difference (ANOVA test, $P < 0.05$) in DOC concentrations between cultures and the control. In general, DOC mean concentrations were highest at the beginning (310 μM) and the end (356 μM) of the experiments and lowest during the mid-exponential growth phase (253 μM). The high DOC concentrations found in the cultures at the beginning of the experiments are thought to be due to organic carbon in the *E. huxleyi* stock culture used to inoculate the experimental flasks, as the initial DOC concentrations in the control are lower. In future work, it is recommended that sterile filtered stock culture is added to the control flask to account for the addition of DOM that inevitably accompanies inoculation of media with stock culture. Furthermore, lower overall DOM levels may be obtained by using artificial seawater. The decrease of DON concentrations at the end of the experiments suggested phytoplankton DON uptake after nitrate was depleted. Ammonium (results not shown) remained low (≤ 5 μM) throughout the experimental period in the cultures.

The release or depletion rates of DOM were calculated as the net change in concentration over the selected period in days, normalized to average cell count (Table 1). In general, higher release rates of DON by *E. huxleyi* were detected during the early and mid-exponential growth. However, a contrasting trend was observed for DOC with higher rates of release at the middle and end of exponential growth. The percentage release of DON relative to the nitrate uptake over each sampling interval was calculated by using the changes of nitrate and DON concentrations as shown in Table 1. Higher DON release rates relative to nitrate uptake were recorded during the exponential growth phase with the highest release rates (62%) at the early part of the exponential growth (days 0–5) and lowest (38%) at the end of the sampling period.

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Table 1. Rates of DOM release/depletion during growth of *Emiliana huxleyi* in nitrate culture media.

Time interval	DOC			DON			Nitrate	DON release rate relative to nitrate uptake
(d)	(μM)	(μM day^{-1})	(μmol cell^{-1} day^{-1})	(μM)	(μM day^{-1})	(μmol cell^{-1} day^{-1})	(μM)	(%)
0–5	−57	−15.8	-1.77×10^{-7}	+15.2	+3.0	$+3.88 \times 10^{-8}$	−24.5	62
5–7	+48	+24.0	$+1.12 \times 10^{-7}$	+15.2	+7.6	$+3.53 \times 10^{-8}$	−40.5	38
7–14	+55	+7.9	$+1.65 \times 10^{-8}$	− 16.7	−2.4	-5.00×10^{-9}	−38.3	44

+, release; −, depletion.

These results clearly indicate that there was an increase of DON and DOC concentrations with time, with the highest concentrations of DON (33.5 μM) and DOC (356 μM) seen during the middle and late exponential growth phase respectively. Since the cultures were axenic, DOM was produced directly by release from phytoplankton. Furthermore, increases in DOM were not accompanied by any marked decrease in cell numbers suggesting that this increase was mainly due to release of catabolized N compounds rather than from cell death. In terms of stoichiometry, the DOC:DON ratio was 13.6, 9.0 and 21.2 on days 5, 7 and 14 respectively showing non-Redfield release of DOC and DON i.e. C:N = 6.6 (Redfield *et al.*, 1966) with potential decoupling of these cycles with respect to DOM.

The DOM release results presented can be compared with other studies which have also observed a similar trend of DOM release in cultures (Newell *et al.*, 1972; Myklestad *et al.*, 1989; Collos, 1992; Collos *et al.*, 1992; Pujo-Pay *et al.*, 1997; Aluwihare & Repeta, 1999; Engel *et al.*, 2004; Mulholland *et al.*, 2004). However, most of the studies used different species, mainly *Phaeodactylum tricornutum* and *Dunaliella tertiolecta*, with only Aluwihare & Repeta (1999) and Engel *et al.* (2004) using *E. huxleyi* for DOM studies, although Aluwihare & Repeta (1999) focused on the chemical characteristics of extracellular DOC produced. In the experiment of Aluwihare & Repeta (1999), bacteria were also present throughout the growth period and hence the DOC release would be expected to be influenced by bacterial DOM uptake. Engel *et al.* (2004) showed high variability in DOC concentrations released by *E. huxleyi*.

Other studies have compared nutrient uptake and DON release in culture experiments. Pujo-Pay *et al.* (1997) showed for *P. tricornutum* and *D. tertiolecta*, that less than 10% of the nitrate uptake was released or excreted as DON with release rates of DON from 10.4 to 13.3 nmol N L^{-1} h^{-1}. Experiments using a range of species also indicated that excreted or released DON could represent 63–75% of nitrate or DIN uptake (Collos, 1992; Collos *et al.*, 1992). In this study an average of 48% of nitrate uptake was released as DON. Collos *et al.* (1992) recorded higher values of 39% and 65% during the exponential growth of *Synedra planctonica*, while lower release was found for *D. tertiolecta* (range of 7–25%, mean 11%) and *Chroomonas* sp. (range of 6–50%, mean 22%), for which the highest release was recorded during the stationary growth period (Newell *et al.*, 1972). High release ranging from 0–78% (mean 30%) relative to nitrate uptake was also observed in *Thalassiosira fluviatilis* (Conover, 1975). This present study therefore provides an estimate of the amount of DON production that might be released during active phytoplankton growth, with DON release up to ~60%.

There is no general agreement on which phase of growth exhibits the highest rates of DOM release. Some studies have observed highest release rates in the stationary phase during the decomposition of the algal cells (Newell *et al.*, 1972; Conover, 1975), while others have shown that they occur during the exponential growth phase (Myklestad *et al.*, 1989; Collos *et al.*, 1992; Aluwihare & Repeta, 1999). The present study indicates high release rates of DOM were found during the early and mid-exponential growth, consistent with the hypothesis that healthy living cells contribute to DOM release. However, it should be noted that the relatively short-term experiments (<14 days) in this study mean that the later stages of growth cannot be considered.

Although this culture study used significantly higher nitrate concentrations (~100 μM) than generally found in the marine environment, blooms of *E. huxleyi* are potentially an important source of DOC and DON. This release was also shown to be decoupled during the growth of *E. huxleyi* as has been implied by field studies (Painter *et al.*, 2007) and may have important large scale biogeochemical implications (Arrigo, 2005).

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