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Effects of varying growth irradiance and nitrogen sources on calcification and physiological performance of the coccolithophore *Gephyrocapsa oceanica* grown under nitrogen limitation

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Abstract

Gephyrocapsa oceanica is a widespread species of coccolithophore that has a significant impact on the global carbon cycle through photosynthesis and calcium carbonate precipitation. We investigated combined effects of light (50 μmol m^{-2} s^{-1}, 190 μmol m^{-2} s^{-1}, and 400 μmol m^{-2} s^{-1}) and the nitrogen sources NO_3^- and NH_4^+ on its physiological performance under nitrogen-limited conditions. The specific growth rate was highest at the mid-range light level of 190 μmol m^{-2} s^{-1}, where it was further accelerated by NH_4^+ relative to NO_3^-. There were no significant growth rate differences between NO_3^-- and NH_4^+-grown cells at the two light levels either above or below this optimum irradiance. Cellular particulate organic carbon (POC) and nitrogen (PON) content were not significantly affected by different light intensities and nitrogen sources. However, both the cellular particulate inorganic carbon (PIC) content and the PIC to POC ratio were greatly decreased by increased light levels, and were further decreased by NH_4^+ only at the highest light level. Non-photochemical quenching (NPQ) increased with increasing light intensity, and was higher in NO_3^- rather than in NH_4^+-grown cells at medium and high light intensities. Our results demonstrate that under low, relatively realistic oceanic nitrogen concentrations, increasing light intensity and the replacement of NO_3^- by NH_4^+ would have a significant negative effect on the calcification of the coccolithophore *G. oceanica*. If these findings are also applicable to other coccolithophore species, the future ocean carbon cycle may be greatly affected.

Marine phytoplankton play an important role in the ocean's food webs and global biogeochemical cycles, and one group that receives much attention in this regard is the coccolithophores (Haptophyta, Prymnesiophyceae). Coccolithophores are unicellular marine algae that have the ability to form delicate calcite scales. The most abundant and cosmopolitan species are *Gephyrocapsa oceanica* and *Emiliania huxleyi* (Paasche 2002). These two species regularly form vast blooms in late spring and mid-summer, and have a significant impact on the carbon cycle through photosynthesis and calcification (Brown and Yoder 1994; Brown 1995; Raitsos et al. 2006). The relative degree of calcification and photosynthesis in the euphotic layer determines the biologically mediated exchange of carbon dioxide (CO_2) between the atmospheric and oceanic carbon reservoirs (Sigman et al. 2010), making estimation of these two processes important to our understanding of the dynamics of the global carbon cycle.

Due to human activities CO_2 is accumulating in the atmosphere, resulting in warming of both ocean and terrestrial ecosystems as more infrared radiation is trapped (Sabine et al. 2004). Ocean warming as well as climate change-driven ocean freshening (ice melting, rain) can make the upper mixed layer become shallower (Hays et al. 2005; Doney 2006; Capotondi et al. 2012), leading to enhanced average levels of photosynthetic active radiation (PAR) experienced by marine phytoplankton. The upward transport of nutrients from deeper, nutrient rich waters will also be decreased by enhanced stratification, leading to more marked and frequent nutrient limitation (Cermeño et al. 2008; Gao et al. 2012). At the same time, surface ocean acidity is increasing as a result of more CO_2 dissolving in sea water, a process defined as ocean acidification (Doney et al. 2009). Recent studies suggest that the nitrogen cycle may respond strongly to the increasing of pCO_2, and this change may decrease the NO_3^-/NH_4^+ ratio in surface seawater (Hutchins et al. 2009; Beman et al. 2011), due to a combination of increased nitrogen fixation and decreased ammonia oxidation rates under low pH and elevated CO_2 (Ward 1987; Hutchins et al. 2015).

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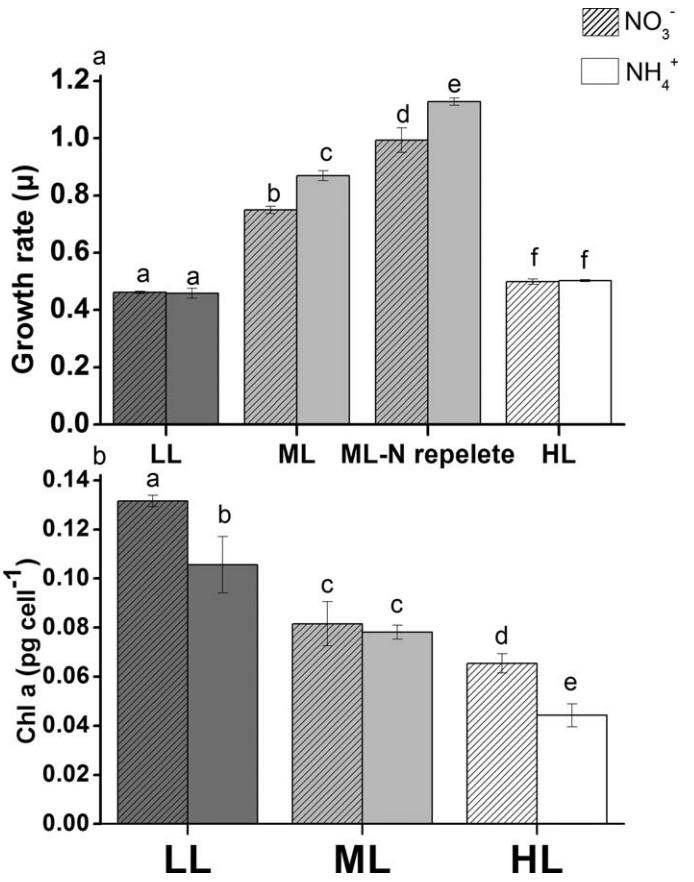

Fig. 1. Growth rate (a) and cellular Chl *a* content (b) in NO_3^- and NH_4^+-grown cultures under nitrogen limited conditions at LL (50 μmol m^{-2} s^{-1}), ML (190 μmol m^{-2} s^{-1}), and HL (400 μmol m^{-2} s^{-1}). The growth rate (a) also includes data from cultures grown at ML with replete nitrogen sources (ML-N replete) for comparison. The different letters above the bars indicate significant differences among the treatments ($p < 0.05$). The values are the means and error bars are standard deviations for triplicate cultures at each treatment.

In the ocean the availability of the inorganic nitrogen sources NO_3^-, NH_4^+, and NO_2^-, is a crucial limiting factor on marine phytoplankton growth and physiological performance (Ryther and Dunstan 1971). Although NO_3^- concentrations can be very high in upwelling regions, in oligotrophic areas NO_3^- is supplied relatively slowly to the upper mixed layer, and so recycled NH_4^+ is often the primary nitrogen source for phytoplankton (Holmes et al. 1999). Generally, the fully reduced form of nitrogen in NH_4^+ is preferentially taken up and assimilated relative to fully oxidized NO_3^- (Dugdale and Goering 1967). The energetic cost for the uptake of NO_3^- is higher relative to NH_4^+ uptake (Falkowski and Stone 1975), and following uptake NH_4^+ can be assimilated into amino acids directly while NO_3^- must first be reduced to NO_2^- and then to NH_4^+ (Levasseur et al. 1993). Consequently, cells grown on NO_3^- may have slower growth rates compared to growth using NH_4^+. To maintain growth rates, NO_3^- -grown cells may compensate for its higher reductant and energy requirements in several ways including changing chemical composition, chlorophyll concentration and electron transport rates (Levasseur et al. 1993). As light is the major energy source for phytoplankton, their physiological performance may be more seriously restricted with NO_3^- compared to NH_4^+ when growing under limiting light conditions, while saturating light may relieve this restriction (Thompson et al. 1989).

Coccolithophores depend on light energy not only for photosynthesis, but also for calcification. Lefebvre et al. (2012) reported that NH_4^+ assimilation under nitrogen replete conditions depresses calcification and alters coccolith morphology in *E. huxleyi*. However, in their study, only one light intensity was used. It is currently not known whether this response is universal across a range of light levels, and how any consequent changes in calcification rates could influence photosynthetic performance under different irradiances and nitrogen sources. Given this, it is possible that future ocean environmental conditions including lower nutrient availability, higher ratios of NH_4^+ to NO_3^-, and intensified light levels may have a more significant impact on coccolithophores than on other phytoplankton. Although a number of studies have investigated the effect of light and nitrogen on coccolithophores, they mainly focused on *E. huxleyi* (Merrett et al. 1993; Paasche 2002; Langer and Benner 2009; Kaffes et al. 2010). To our knowledge, there still has been no work to study the interactions between varying light and nitrogen sources on *G. oceanica* under nitrogen limiting conditions. The general objective of this study was to determine how growth limited by NO_3^- or NH_4^+ under three different light conditions affects photosynthesis and calcification of the ecologically important coccolithophore species *G. oceanica*.

Materials and methods

Phytoplankton culturing

G. oceanica (NIES-1318) originally isolated from the East China Sea was obtained from the National Institute for Environmental Studies in Japan. Cultures were grown in artificial seawater enriched with Aquil nutrients (Sunda et al. 2005), except that N was supplied as either 5 μmol L^{-1} NO_3^- or as 5 μmol L^{-1} NH_4^+. Cells were also cultured in replete N of 100 μmol L^{-1} NO_3^- and NH_4^+ at PFD of 190 μmol m^{-2} s^{-1} (the medium light level) to measure maximum growth rates. The low experimental nitrogen concentration (5 compared to 100 μmol L^{-1}) significantly reduced the growth rate of the cells ($p < 0.01$, Fig. 1a), and so represents a nitrogen limited condition (Fig. 1a). Cultures were illuminated on a light/dark cycle of 12/12 h with cool-white fluorescent lamps at photon flux densities (PFDs) of 50 (LL, low light, unsaturated), 190 (ML, medium light, saturated) and 400 μmol m^{-2} s^{-1} (HL, high light, inhibited).

The following experimental treatments were used in a triplicated factorial matrix of the three light levels and two nitrogen sources: LL with NO_3^-, LL with NH_4^+, ML with NO_3^-, ML with NH_4^+, HL with NO_3^-, and HL with NH_4^+. The cells were grown semi-continuously at 20°C in a CO_2 chamber (HP1000 G-D, Ruihua Instrument and Equipment, Wuhan, China). The culture media was pre-equilibrated with an ambient CO_2 concentration of 400 ppm and the cultures were maintained in tightly closed polycarbonate bottles without any head gas space, to prevent CO_2 gas exchange. Dilutions were carried out within 48 h to ensure cell concentrations did not exceed 8×10^4 cells mL^{-1} and that they were continuously in exponential growth phase. For dilutions, the culture bottles were not moved overnight so that most cells sank to the bottom. The supernatant medium was then removed carefully by siphoning just before the start of the light period, and a small volume of concentrated cells on the bottom was added to the new culture medium at an initial calculated cell concentration of 3×10^4 cells mL^{-1}. The cell concentration was measured at the midpoint of the light period after dilution (t_0), and measured again 1-d later at the same time (t_1), before the start of another dilution cycle. Cells were acclimated to each light and nitrogen combination for more than 15 generations before being used in the experiments.

Measurements and analysis

Growth rates and chlorophyll *a*

Cell concentration was measured using a particle counter (Z2, Beckman Instruments, FL). Determinations of growth rates were based on the change in cell numbers every 24 h, and were calculated according to the equation: $\mu = (\ln N_1 - \ln N_0)/(t_1 - t_0)$, where N_0 and N_1 were the cell concentrations at t_0 and t_1, respectively. For chlorophyll *a* (Chl *a*) content determination, cells were filtered onto Whatman GF/F filters at time t_1, extracted overnight in absolute methanol, and centrifuged for 10 min at $6000 \times g$. Absorbance of the supernatant was then measured from 200 nm to 800 nm with a scanning spectrophotometer (DU 800, Beckman Coulter). The Chl *a* concentration in the supernatant was calculated according to Porra (2002), where [Chl *a*] ($\mu g\ mL^{-1}$) = $16.29 \times (A_{665} - A_{750}) - 8.54 \times (A_{652} - A_{750})$.

C and N analysis

Samples taken at time $t_1$were filtered onto precombusted (450°C for 5 h) Whatman GF/F filters (25 mm) and frozen at −20°C. For particulate organic carbon (POC) analysis, inorganic carbon was removed by fuming the filters over HCl for 12 h while samples for total particulate carbon (TPC) analysis were not treated with HCl. All samples were dried for 12 h and analyzed using a Perkin Elmer Series II CHNS/O Analyzer 2400 instrument (Perkin Elmer Waltham, MA). PIC was determined as the difference between particulate organic carbon (POC) and total particulate carbon (TPC). The production rates (P) of particulate organic nitrogen (PON) or POC were calculated as: P (pg $cell^{-1}$ d^{-1}) = specific growth rate μ (d^{-1}) × cellular PON or POC content (pg $cell^{-1}$).

Fluorescence parameters

Samples were taken at time t_1 and analyzed with a XE-PAM (Walz, Germany). For maximum quantum yield (F_v/F_m), cells were first dark-adapted for 15 min, the measuring light intensity was set at 0.3 μmol photons m^{-2} s^{-1}, and the saturating pulse was set at 5,000 μmol photons m^{-2} s^{-1} (0.8 s). For effective quantum yield (F'_v/F'_m), the actinic light was set at 76 μmol m^{-2} s^{-1}, 200 μmol m^{-2} s^{-1}, and 533 μmol m^{-2} s^{-1} for LL, ML and HL treatments, respectively, which was close to the light level in each treatment. The non-photochemical quenching (NPQ) was calculated as, $NPQ = (F_m - F'_m)/F'_m$.

Data analysis

One-way and two-way analysis of variance (ANOVA) were used to establish differences among the treatments ($p = 0.05$). Unless otherwise indicated, all reported p value refers to one-way ANOVA, LSD (least significant difference).

Results

Growth rate and Chl *a* content

To determine that the 5 μM nitrogen concentration was limiting for *G. oceanica*, we also cultured the cells under replete nitrate and ammonium concentrations (100 μM). With replete nitrate and ammonium, the growth rates were only 32.9% and 29.8% higher at ML than with limiting concentrations at ML ($p < 0.01$, Fig. 1a). In the ML nitrogen-replete cultures, growth rate was also 13% higher in NH_4^+ than in NO_3^- cultures ($p < 0.01$, Fig. 1a). Under nitrogen-limited conditions, the growth rates were significantly further reduced by LL and HL. Nitrogen-limited growth rates were slightly higher at HL than LL both in NO_3^- ($p < 0.01$) and NH_4^+ cultures ($p < 0.05$), but there were no significant differences between the two nitrogen sources within these two light levels ($p > 0.05$). However, in the ML nitrogen-limited treatment, the growth rate of NH_4^+-grown cells was 16% higher than that of the NO_3^--grown ones ($p < 0.01$, Fig. 1a). There were significant interactive effects between light and nitrogen source on growth rate (two-way ANOVA, $F_{2,12} = 48.7$, $p < 0.01$).

The cellular Chl *a* content for both nitrogen sources was highest at LL, and decreased with increasing light levels. This value was significantly higher in NO_3^- cultures than NH_4^+ cultures both at LL ($p < 0.05$, Fig. 1b) and HL ($p < 0.01$). However, at ML there was no significant difference for cellular Chl *a* content between different nitrogen sources ($p > 0.05$).

C and N cellular quotas and production rates

The cellular POC values were not significantly different between NO_3^- and NH_4^+ treatment at all the three light levels

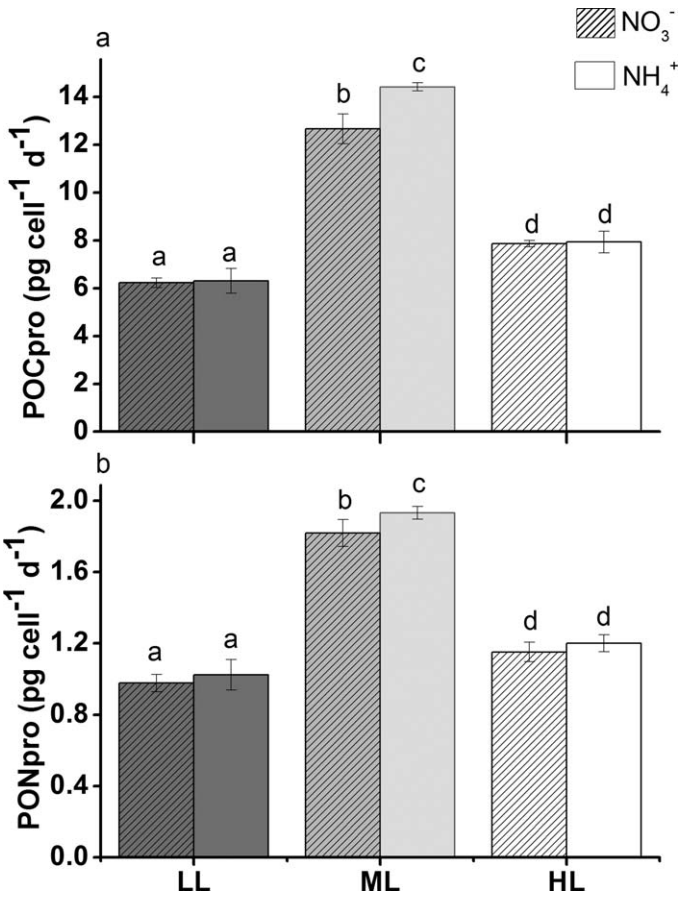

Fig. 2. POC production rate (a) and PON production rate (b) in NO_3^- and NH_4^+-grown cultures under nitrogen limited conditions at LL (50 μmol m^{-2} s^{-1}), ML (190 μmol m^{-2} s^{-1}), and HL (400 μmol m^{-2} s^{-1}). The different letters above the bars indicate significant differences among the treatments ($p < 0.05$). The values are the means and error bars are standard deviations for triplicate cultures at each treatment.

(Table 1). However, cellular POC was significantly lower at LL than at ML and HL in both the NO_3^- and NH_4^+ treatments. The production rate of POC showed a similar trend with that of cellular POC quotas at LL and ML, but HL enhanced POC production rate by about 26% in both NO_3^- cultures and NH_4^+ cultures relative to LL, respectively (Fig. 2a). At ML, the POC production rate was greatly increased compared to LL and HL ($p < 0.01$) and was further increased by NH_4^+ ($p < 0.01$, Fig. 2a).

Cellular PON quotas were slightly but significantly higher in NO_3^- -grown cells than NH_4^+ -grown ones only at ML ($p < 0.01$). There were significant lower PON quotas at LL compared to at ML and HL in NO_3^- -grown cells ($p < 0.05$), but no significant difference existed between light levels in NH_4^+ -grown cells (Table 1). The production rate of PON showed a similar trend with that of POC, in that the value was greatly increased at ML ($p < 0.01$), and there was a significant difference for PON production rate between NO_3^-- and NH_4^+-grown cells only at ML, but not at the other two light levels ($p < 0.01$, Fig. 2b). The C/N ratio was highest at ML ($p < 0.01$ for NH_4^+ cultures but no significant difference for NO_3^- cultures), and the value was significantly lower in NO_3^- cultures than NH_4^+ cultures ($p < 0.05$, Table 1), mainly due to higher cellular N quotas in NO_3^--grown cells at ML. There was no significant difference in C/N ratios between the different nitrogen sources at LL and HL ($p > 0.05$, Table 1).

Table 1. Cellular POC, PON, and POC/PON ratio in NO_3^- and NH_4^+-grown cultures under nitrogen limited conditions at LL (50 μmol m^{-2} s^{-1}), ML (190 μmol m^{-2} s^{-1}) and HL (400 μmol m^{-2} s^{-1}). Shown are the means and standard deviations for triplicate cultures at each treatment. The exponent letters on the values indicate significant differences among the treatments ($p < 0.05$).

	POC (pg $cell^{-1}$)	PON (pg $cell^{-1}$)	POC/PON
LL with NO_3^-	13.50 ± 0.37^a	2.12 ± 0.09^a	7.44 ± 0.38^a
LL with NH_4^+	13.74 ± 0.71^a	2.23 ± 0.11^{ab}	7.18 ± 0.06^a
ML with NO_3^-	16.89 ± 0.55^b	2.43 ± 0.06^b	8.12 ± 0.09^a
ML with NH_4^+	16.61 ± 0.48^b	2.23 ± 0.01^a	8.71 ± 0.23^b
HL with NO_3^-	15.77 ± 0.47^b	2.31 ± 0.15^b	7.98 ± 0.28^a
HL with NH_4^+	15.79 ± 0.79^b	2.39 ± 0.08^b	7.89 ± 0.17^a

The cellular PIC content decreased significantly at ML compared to LL by more than 2.1 and 1.8 fold ($p < 0.01$) in NO_3^- and NH_4^+-grown cultures, respectively, and was even further decreased under HL ($p < 0.01$, Fig. 3a). The PIC to POC ratio (PIC/POC) exhibited the same trend as cellular PIC, with values that were 2.7 and 2.3 times higher in NO_3^- and NH_4^+ cultures, respectively, under LL compared to those at ML ($p < 0.01$). PIC/POC decreased by a further 1.4 and 2.4 fold in NO_3^- and NH_4^+ cultures at HL relative to ML ($p < 0.01$, Fig. 3b). For PIC production rate, there was no significant difference between the LL and ML treatment ($p > 0.05$, Fig. 3c). However, the PIC production rate was drastically lower at HL than at either LL or ML ($p < 0.01$). There were significant interactive effects between light and nitrogen source on the production rate of PIC (two-way ANOVA, $F_{2,12} = 5.3$, $p = 0.03$). At ML, the PIC production rate in NH_4^+ cultures was 22.5% higher than in NO_3^- cultures ($p < 0.01$), while in contrast, this value was 44.3% lower in NH_4^+ cultures than in NO_3^- cultures at HL ($p < 0.05$, Fig. 3c). There were no significant differences in PIC production rates between NO_3^-- and NH_4^+-grown cells at LL ($p > 0.05$).

Fluorescence parameters

The maximum quantum yield (F_v/F_m) and effective quantum yield (F'_v / F'_m) were similar in the LL and ML treatment ($p > 0.05$), but were significantly reduced at HL (Fig. 4a,b). At LL and ML, the F_v/F_m was slightly but significantly higher in NO_3^- cultures than NH_4^+ cultures ($p < 0.05$, Fig. 4a). The F'_v / F'_m was also slightly but significantly higher in NO_3^- cultures

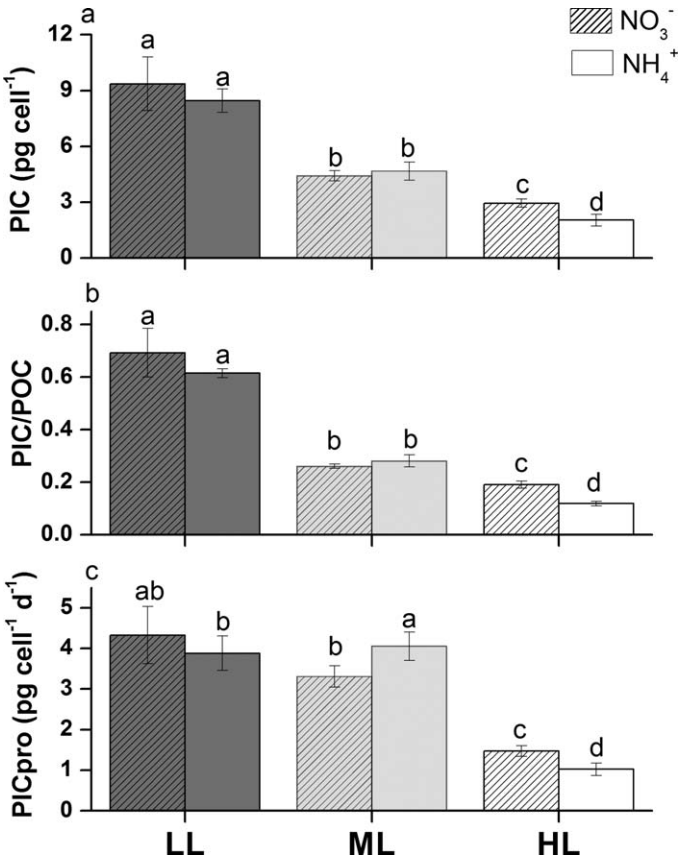

Fig. 3. Cellular PIC content (a), PIC/POC ratio (b), and PIC production rate (c) in NO_3^- and NH_4^+-grown cultures under nitrogen limited conditions at LL (50 μmol m^{-2} s^{-1}), ML (190 μmol m^{-2} s^{-1}), and HL (400 μmol m^{-2} s^{-1}). The different letters above the bars indicate significant differences among the treatments ($p < 0.05$). The values are the means and error bars are standard deviations for triplicate cultures at each treatment.

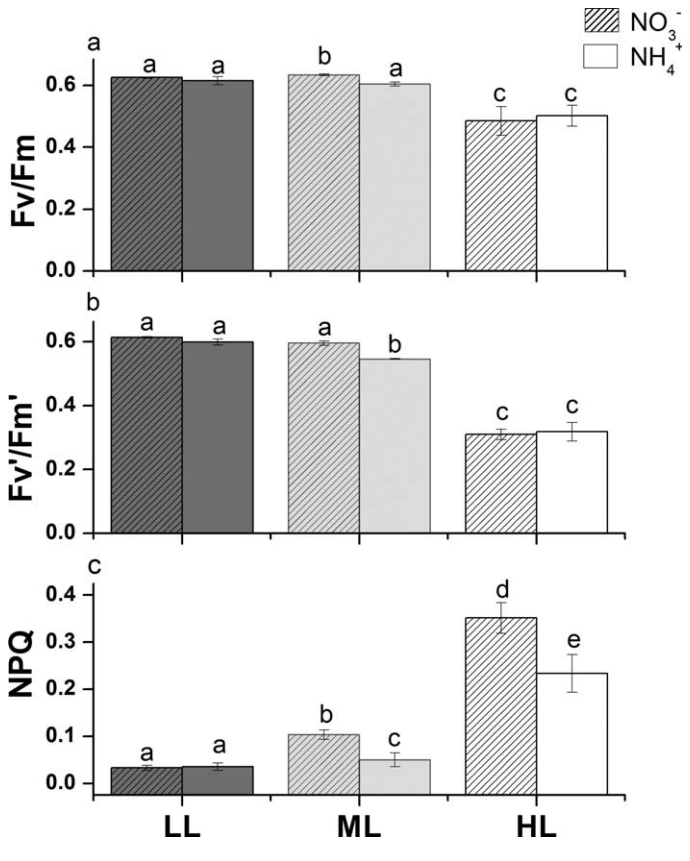

Fig. 4. F_v/F_m (a), F_v' / F_m' (b), and NPQ (c) in NO_3^- and NH_4^+-grown cultures under nitrogen limited conditions at LL (50 μmol m^{-2} s^{-1}), ML (190 μmol m^{-2} s^{-1}), and HL (400 μmol m^{-2} s^{-1}). The different letters above the bars indicate significant differences among the treatments ($p < 0.05$). The values are the means and error bars are standard deviations for triplicate cultures at each treatment.

than NH_4^+ cultures at ML ($p < 0.01$, Fig. 4b), but for the other two light levels, no significant differences existed between different nitrogen sources ($p > 0.05$). The NPQ was low at LL, and was increased stepwise at ML and HL, being 3.4 fold and 4.6 fold higher at HL than ML in NO_3^- and NH_4^+ cultures, respectively ($p < 0.01$, Fig. 4c). For the two different nitrogen sources, the NPQ was 106% and 50% higher in NO_3^- cultures than in NH_4^+ cultures at ML and HL, respectively ($p < 0.01$, Fig. 4c).

Discussion

Although the reduced nitrogen source we used was NH_4^+, in fact, about 10% of the total ammonia is present as the un-ionized form NH_3 at the sea water pH of 8.0. Both NH_4^+ and NH_3 can be toxic to phytoplankton cells above a certain concentration, but NH_3 is considered to be the most toxic form because it is uncharged and lipid soluble and easily diffuses across membranes. Collos and Harrison (2014) noted that the tolerance for Prymnesiophyceae to highly toxic NH_4^+ was 2300 μmol L^{-1}. Lefebvre et al. (2012) also reported that NH_4^+ only became toxic to *E. huxleyi* at concentrations of 1 mmol L^{-1} and above. In comparison, the 5 μmol L^{-1} NH_4^+ and 100 μmol L^{-1} NH_4^+ concentration used in this study was unlikely to have been toxic to *G. oceanica*. After our *G. oceanica* cultures acclimated for more than 15 generations of near steady-state growth, growth rates were highest at the middle light level (ML) but almost equally reduced under both low (LL) and high (HL) light conditions, due to light limitation and photo-inhibition, respectively. NH_4^+ promoted growth rates relative to NO_3^- at this optimal light level (ML), regardless of whether nitrogen was limiting or not. Under low light conditions (LL), however, there was no difference in growth rates between nitrogen-limited NO_3^-- and NH_4^+-grown cells. This suggests that despite higher levels of Chl *a* per cell under LL the cultures were energetically limited by light availability, thus neutralizing any advantage of growth on NH_4^+. Likewise, growth rates were identical between NO_3^- and NH_4^+ cultures at high light levels. *G. oceanica* cells were clearly photo-inhibited under this high irradiance, as evidenced by the significantly decreased F_v/F_m

and F'_v/F'_m. As a result of these two differing light response mechanisms, there was no energetic advantage at either LL or HL for cells growing on NH_4^+ compared to NO_3^-.

Before our experiment started, we hypothesized that the lower amount of photosynthetically derived reductant required for NH_4^+ assimilation in comparison to NO_3^- assimilation would allow NH_4^+-grown cells to grow faster and synthesize more organic carbon and nitrogen, especially at LL. However, there was no difference in cellular POC and PON quota between NO_3^- and NH_4^+ cultures between the three light levels, although POC and PON production rates were higher in NH_4^+-grown compared to NO_3^- cultures at ML. Shi et al. (2015) examined interrelated effects of nitrogen source, light and CO_2 on the diatom *Thalassiosira pseudonana* and incorporated them into an energetic budget to model its photosynthetic response. They found that the predicted energetic differences between NO_3^- and NH_4^+-grown cultures were in good agreement with observations at low light, but that predicted and observed responses did not match well at high light.

Other previous studies have also found that growth on NH_4^+ rather than NO_3^- provided phytoplankton with less energetic advantage than expected, but especially when light is severely limiting. For example, in the same diatom species *T. pseudonana* under low light, NO_3^--grown cells have equal growth rates, Chl *a* cell^{-1}, nitrogen quotas and equal or greater carbon quotas compared to NH_4^+-grown cells. However, under higher light, NO_3^--grown cells exhibit reduced growth rates and carbon quotas (Thompson et al. 1989). Levasseur et al. (1993) found that at saturating photon flux levels when energy is not limiting, the utilization of NH_4^+ compared to NO_3^- resulted in higher growth rates and N quotas in the diatom *Chaetoceros* sp., whereas at subsaturating light levels, NH_4^+- and NO_3^--grown cells had similar growth rates and C and N quotas.

These prior results demonstrate that although in theory NH_4^+ could promote phytoplankton growth compared to NO_3^-, actual observations can differ. One potential explanation for this discrepancy may be that although the total organic carbon and nitrogen were the same between NO_3^-- and NH_4^+-grown cells under LL and HL, the specific organic compounds produced may be different. Because lipids have the highest energy densities among organic molecules, it has been suggested that in the absence of more energetically demanding NO_3^- assimilation phytoplankton synthesize more fatty acids as a sink for excess absorbed light energy (Frada et al. 2013). In support of this hypothesis, our ML cells had significantly higher POC : PON ratios during NH_4^+-supported growth than with NO_3^- as a sole nitrogen source, consistent with production of more carbon-rich lipids rather than nitrogen-rich proteins. The changing of synthesized organic compounds from proteins to lipids was also observed in *E. huxleyi* when growing conditions were changed (Kaffes et al. 2010).

Few studies have examined nitrogen source effects on ecologically important coccolithophores, particularly with regards to how they may interact with other environmental factors to influence physiological performance. Lefebvre et al. (2012) reported that when NO_3^- was partially replaced by NH_4^+ under nitrogen replete conditions, the volume-normalized cellular POC content was increased in *E. huxleyi* strain CCMP 371 under high CO_2, but this effect of NH_4^+ was insignificant under low CO_2 conditions. However, because this previous study used nutrient-replete cultures grown with mixed nitrogen sources under two CO_2 levels, their results are not directly comparable to ours using nitrogen-limited cultures with either NO_3^- or NH_4^+ as a sole N source under ambient CO_2 concentrations.

In our study, we observed a large reduction in cellular PIC quota and PIC/POC ratio with increasing light intensity, regardless of the nitrogen source used. This is surprising, as calcification in coccolithophores is an energy-requiring process, and so is light-dependent in some cases. This has been shown in several laboratory studies with *E. huxleyi*, where calcification was strongly dependent on the irradiance, with higher light levels yielding more calcification (Nimer and Merrett 1993; Trimborn et al. 2007). The results of many in situ studies also coincided with these laboratory culture results, in that cellular calcification rates were generally high at the ocean's surface, and decreased sharply in subsurface layers (Fernandez et al. 1993; Wal et al. 1995).

Although most studies agree that calcification often increases with light intensity, there are also a number of exceptions that have been reported. A field study conducted in the Bay of Biscay found that an *E. huxleyi* population shifted seasonally between lightly calcified forms in summer (> 90%) and heavily calcified forms in winter (> 90%), even although the deepest mixing and the lowest surface incident light density and hence, the lowest average mixed layer irradiances occurred in winter (Smith et al. 2012). Xu and Gao (2015) also reported reduced cellular PIC and PIC/POC in cultured *E. huxleyi* when the light level was increased from 300 μmol photons m^{-2}s^{-1} to 500 μmol photons m^{-2}s^{-1}. Feng et al. (2008) observed in a culture study with another *E. huxleyi* strain that the PIC/POC ratio decreased significantly at HL compared to LL. Previous work suggests that the saturating irradiance for photosynthesis in *E. huxleyi* is much higher than that for calcification (Balch et al. 1992; Bleijswijk et al. 1994). In the Feng et al. (2008) study, decreased calcification under HL was attributed to the irradiances used in the incubations. They suggested calcification may have already been photoinhibited while photosynthesis was not, leading to markedly decreased cellular PIC quotas and PIC/POC ratios compared to the LL cultures. Paasche (2002) had much earlier reviewed previous studies investigating the relationship between irradiance and calcification in *E. huxleyi*, mainly by short-term ^{14}C uptake experiments, and drew the conclusion that coccolith production in *E. huxleyi* was

strongly light-dependent. However, they also demonstrated that there was ample evidence that *E. huxleyi* could form coccoliths in the dark. So it seems that calcification is not always light-dependent. Much more work is needed to reveal the mechanism of calcification.

Cellular PIC content and PIC/POC also decreased significantly in NH_4^+-grown cultures compared to NO_3^--grown cultures under HL, a phenomenon which was also observed by Lefebvre et al. (2012). A possible explanation could be the intracellular pH differences created by the different nitrogen sources when they are assimilated. Calcification uses HCO_3^- as the main dissolved inorganic carbon (DIC) source (Sekino and Shiraiwa 1994; Buitenhuis et al. 1999; Berry et al. 2002; Rickaby et al. 2010; Bach et al. 2015), and when HCO_3^- is converted to CO_3^{2-} to form calcite crystals in the coccolith vesicle, excess H^+ is created. This is an important reason for the commonly observed reduced calcification of coccolithophores under ocean acidification (Raven and Crawfurd 2012).

As the assimilation of NH_4^+ produces excess H^+ ions, whereas assimilation of NO_3^- produces OH^- (Raven 1986), this effect may be reflected in our NPQ values. The major determinant of NPQ in plants is the need to build up a thylakoid pH gradient to induce qE (Muller et al. 2001). It is possible in our study that production of excess OH^- through the assimilation of N O_3^- increased the pH gradient across the thylakoid membrane, while NH_4^+ assimilation reduced the pH gradient, thus leading to higher NPQ in NO_3^--grown cells. As a result, the H^+ generated during NH_4^+ assimilation would inhibit the internal conversion from HCO_3^- to H^+ and CO_3^{2-}, thereby the availability of CO_3^{2-} for calcite production is restricted. In contrast, the OH^- generated by NO_3^- assimilation could neutralize the H^+ generated when HCO_3^- dissociates to CO_3^{2-}, thus promoting calcification. However, cellular calcification was decreased by NH_4^+ only under HL. One of the reasons may be that as where calcification rate was drastically reduced under HL, the H^+ and OH^- generated by nitrogen assimilation accounted for a very large proportion of that generated by calcification, so different nitrogen sources had a significant impact on calcification. Additional experiments should be conducted to better understand the impact of different nitrogen sources on coccolithophore calcification.

Our study demonstrates that under realistic oceanic conditions of limiting nitrogen concentrations, the widespread coccolithophore species *G. oceanica* decreases its cellular PIC content and PIC/POC ratio with increasing light intensity. These values are further decreased by the replacement of NH_4^+ with NO_3^- as a nitrogen source under high irradiance. A substantial reduction in oceanic calcification may be expected in view of a more stratified future ocean with increased mean surface light levels, decreased nutrient transport from deeper waters, and an increasing NH_4^+/NO_3^- ratio, especially if these findings are also applicable to other common coccolithophore species. However, as there is likely to be a diversity of species- and strain-specific responses, to more accurately predict the influence of future climate change on oceanic calcification further interactive multiple variable studies are needed using a range of representative coccolithophores.

References

Bach, L. T., U. Riebesell, M. A. Gutowska, L. Federwisch, and K. G. Schulz. 2015. A unifying concept of coccolithophore sensitivity to changing carbonate chemistry embedded in an ecological framework. Prog. Oceanogr. **135**: 125–138. doi:10.1016/j.pocean.2015.04.012

Balch, W. M., P. M. Holligan, and K. A. Kilpatrick. 1992. Calcification, photosynthesis and growth of the bloom-forming coccolithophore, *Emiliania huxleyi*. Cont. Shelf Res. **12**: 1353–1374. doi:10.1016/0278-4343(92)90059-s

Beman, J. M., and others. 2011. Global declines in oceanic nitrification rates as a consequence of ocean acidification. Proc. Natl. Acad. Sci. USA **108**: 208–213. doi:10.1073/pnas.1011053108

Berry, L., A. R. Taylor, U. Lucken, K. P. Ryan, and C. Brownlee. 2002. Calcification and inorganic carbon acquisition in coccolithophores. Funct. Plant Biol. **29**: 289–299. doi:10.1071/PP01218

Bleijswijk, J. D. L. V., R. S. Kempers, M. J. Veldhuis, and P. Westbroek. 1994. Cell and growth characteristics of type A and B of *Emiliania huxleyi* (Prymnesiophyceae) as determined by flow cytometry and chemical analyses. J. Phycol. **30**: 230–241. doi:10.1111/j.0022-3646.1994.00230.x

Brown, C. W. 1995. Global distribution of Coccolithophore blooms. Future Oceanogr. **8**: 59–60. doi:10.5670/oceanog.1995.21

Brown, C. W., and J. A. Yoder. 1994. Coccolithophorid blooms in the global ocean. J. Geophys. Res. **99**: 7467–7482. doi:10.1029/93jc02156

Buitenhuis, E. T., H. J. W. D. Baar, and M. J. W. Veldhuis. 1999. Photosynthesis and calcification by *Emiliania huxleyi* (Prymnesiophyceae) as a function of inorganic carbon species. J. Phycol. **35**: 949–959. doi:10.1046/j.1529-8817.1999.3550949.x

Capotondi, A., M. A. Alexander, N. A. Bond, E. N. Curchitser, and J. D. Scott. 2012. Enhanced upper ocean stratification with climate change in the CMIP3 models. J. Geophys. Res.: Oceans. **117**: article no. C04031, 1–23. doi:10.1029/2011JC007409

Cermeño, P., S. Dutkiewicz, R. P. Harris, M. Follows, O. Schofield, and P. G. Falkowski. 2008. The role of nutricline depth in regulating the ocean carbon cycle. Proc. Natl. Acad. Sci. USA. **105**: 20344–20349. doi:10.1073/pnas.0811302106

Collos, Y., and P. J. Harrison. 2014. Acclimation and toxicity of high ammonium concentrations to unicellular algae. Mar. Pollut. Bull. **80**: 8–23. doi:10.1016/j.marpolbul.2014.01.006

Doney, S. C. 2006. Oceanography: Plankton in a warmer world. Nature **444**: 695–696. doi:10.1038/444695a

Doney, S. C., V. J. Fabry, R. A. Feely, and J. A. Kleypas. 2009. Ocean Acidification:The Other CO2 Problem. Annu. Rev. Mar. Sci. **1**: 69–92. doi:10.4319/lol.2011.rfeely_sdoney.5

Dugdale, R. C., and J. J. Goering. 1967. Uptake of new and regenerated forms of nitrogen in primary productivity. Limnol. Oceanogr. **12**: 196–206. doi:10.4319/lo.1967.12.2.0196

Falkowski, P. G., and D. P. Stone. 1975. Nitrate uptake in marine phytoplankton: Energy sources and the interaction with carbon fixation. Mar. Biol. **32**: 77–84. doi: 10.1007/bf00395161

Feng, Y., and others. 2008. Interactive effects of increased pCO2, temperature and irradiance on the marine coccolithophore *Emiliania huxleyi* (Prymnesiophyceae). Eur. J. Phycol. **43**: 87–98. doi:10.1080/09670260701664674

Fernandez, E., P. Boyd, P. M. Holligan, and D. S. Arbour. 1993. Production of organic and inorganic carbon within a large-scale coccolithophore bloom in the northeast Atlantic Ocean. Mar. Ecol. Prog. Ser. **97**: 271–285. doi: 10.3354/meps097271

Frada, M. J., E. H. Burrows, K. D. Wyman, and P. G. Falkowski. 2013. Quantum requirements for growth and fatty acid biosynthesis in the marine diatom *Phaeodactylum tricornutum* (Bacillariophyceae) in nitrogen repelete and limited conditions. J. Phycol. **49**: 381–388. doi: 10.1111/jpy.12046

Gao, K., E. W. Helbling, D. P. Häder, and D. A. Hutchins. 2012. Responses of marine primary producers to interactions between ocean acidification, solar radiation, and warming. Mar. Ecol.: Prog. Ser. **470**: 167–189. doi: 10.3354/meps10043

Hays, G. C., A. J. Richardson, and C. Robinson. 2005. Climate change and marine plankton. Trends Ecol. Evol. **20**: 337–344. doi:10.1016/j.tree.2005.03.004

Holmes, R. M., A. Aminot, R. Kerouel, B. A. Hooker, and B. J. Peterson. 1999. A simple and precise method for measuring ammonium in marine and freshwater ecosystems. Can. J. Fish. Aquat.Sci. **56**: 1801–1818. doi:10.1139/f99-128

Hutchins, D. A., M. T. R. Mulholl, and F. Fu. 2009. Nutrient cycles and marine microbes in a CO_2-enriched ocean. Oceanography **22**: 128–145. doi:10.5670/oceanog.2009.103

Hutchins, D. A., and others. 2015. Irreversibly increased nitrogen fixation in *Trichodesmium* experimentally adapted to elevated carbon dioxide. Nat. Commun. **6**: 1–7. doi: 10.1038/ncomms9155

Kaffes, A., and others. 2010. Carbon and nitrogen fluxes in the marine coccolithophore *Emiliania huxleyi* grown under different nitrate concentrations. J. Exp. Mar. Biol. Ecol. **393**: 1–8. doi:10.1016/j.jembe.2010.06.004

Langer, G., and I. Benner. 2009. Effect of elevated nitrate concentration on calcification in *Emiliania huxleyi*. J. Nannoplankton Res. **30**: 77–80.

Lefebvre, S. C., and others. 2012. Nitrogen source and pCO2 synergistically affect carbon allocation, growth and morphology of the coccolithophore *Emiliania huxleyi*: Potential implications of ocean acidification for the carbon cycle. Global Change Biol. **18**: 493–503. doi:10.1111/j.1365-2486.2011.02575.x

Levasseur, M., P. A. Thompson, and P. J. Harrison. 1993. Physiological acclimation of marine phytoplankton to different nitrogen sources. J. Phycol. **29**: 587–595. doi: 10.1111/j.0022-3646.1993.00587.x

Merrett, M. J., L. F. Dong, and N. A. Nimer. 1993. Nitrate availability and calcite production in *Emiliania huxleyi* Lohmann. Eur. J. Phycol. **28**: 243–246. doi:10.1080/09670269300650351

Muller, P., X. P. Li, and K. K. Niyogi. 2001. Non-photochemical quenching. A response to excess light energy. Plant Physiol. **125**: 1558–1566. doi:10.1104/pp.125.4.1558

Nimer, N. A., and M. J. Merrett. 1993. Calcification rate in *Emiliania huxleyi* Lohmann in response to light, nitrate and availability of inorganic carbon. New Phytol. **123**: 673–677. doi:10.1111/j.1469-8137.1993.tb03776.x

Paasche, E. 2002. A review of the coccolithophorid *Emiliania huxleyi* (Prymnesiophyceae), with particular reference to growth, coccolith formation, and calcification-photosynthesis interactions. Phycologia **40**: 503–529. doi: 10.2216/i0031-8884-40-6-503.1

Porra, R. J. 2002. The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b. Photosynth. Res. **73**: 149–156. doi:10.1007/1-4020-3324-9_56

Raitsos, D. E., S. J. Lavender, Y. Pradhan, T. Tyrrell, P. C. Reid, and M. Edwards. 2006. Coccolithophore bloom size variation in response to the regional environment of the subarctic North Atlantic. Limnol. Oceanogr. **51**: 2122–2130. doi:10.4319/lo.2006.51.5.2122

Raven, J. A. 1986. Biochemical disposal of excess H^+ in growth plants. New Phytol. **104**: 175–206. doi:10.1111/j.1469-8137.1986.tb00644.x

Raven, J. A., and K. Crawfurd. 2012. Environmental controls on coccolithophore calcification. Mar. Ecol.: Prog. Ser. **470**: 137–166. doi:10.3354/meps09993

Rickaby, R. E. M., J. Henderiks, and J. N. Young. 2010. Perturbing phytoplankton: Response and isotopic fractionation with changing carbonate chemistry in two coccolithophore species. Clim. Past **6**: 771–785. doi: 10.5194/cp-6-771-2010

Ryther, J. H., and W. M. Dunstan. 1971. Nitrogen, phosphorus, and eutrophication in the coastal marine environment. Science **171**: 1008–1013. doi:10.1126/science.171.3975.1008

Sabine, C. L., and others. 2004. The oceanic sink for anthropogenic CO_2. Science **305**: 367–371. doi:10.1126/science.1097403

Sekino, K., and Y. Shiraiwa. 1994. Accumulation and utilization of dissolved inorganic carbon by a marine unicellular coccolithophorid. *Emiliania huxleyi*. Plant Cell Physiol. **35**: 353–361.

Shi, D., and others. 2015. Interactive effects of light, nitrogen source, and carbon dioxide on energy metabolism in the diatom *Thalassiosira pseudonana*. Limnol. Oceanogr. **60**: 1805–1822. doi:10.1002/lno.10134

Sigman, D. M., M. P. Hain, and G. H. Haug. 2010. The polar ocean and glacial cycles in atmospheric CO_2 concentration. Nature **466**: 47–55. doi:10.1038/nature09149

Smith, H. E. K., and others. 2012. Predominance of heavily calcified coccolithophores at low $CaCO_3$ saturation during winter in the Bay of Biscay. Proc. Natl. Acad. Sci. USA **109**: 8845–8849. doi:10.1073/pnas.1117508109

Sunda, W. G., N. M. Price, and F. M. Morel. 2005. Trace metal ion buffers and their use in culture studies. Algal Culturing Techniques **4**: 35–63. doi:10.1007/s13398-014-0173-7.2

Thompson, P. A., M. E. Levasseur, and P. J. Harrison. 1989. Light-limited growth on ammonium vs. nitrate: What is the advantage for marine phytoplankton? Limnol. Oceanogr. **34**: 1014–1024. doi:10.4319/lo.1989.34.6.1014

Trimborn, S., G. Langer, and B. R. Rost. 2007. Effect of varying calcium concentrations and light intensities on calcification and photosynthesis in *Emiliania huxleyi*. Limnol. Oceanogr. **52**: 2285–2293. doi:10.4319/lo.2007.52.5.2285

Wal, P. V. D., R. S. Kempers, and M. J. W. Veldhuis. 1995. Production and downward flux of organic matter and calcite in a North Sea bloom of the coccolithophore *Emiliania huxleyi*. Mar. Ecol. Prog. Ser. **126**: 247–265. doi:10.3354/meps126247

Ward, B. B. 1987. Kinetic studies on ammonia and methane oxidation by Nitrosococcus oceanus. Arch. Microbiol. **147**: 126–133. doi:10.1007/BF00415273

Xu, K., and K. Gao. 2015. Solar UV Irradiances Modulate Effects of Ocean Acidification on the Coccolithophorid Emiliania huxleyi. Photochem. Photobiol. **91**: 92–101. doi:10.1111/php.12363

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Conflict of Interest

None declared.

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