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Interaction of nitrogen status and UVB sensitivity in a temperate phytoplankton assemblage

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ABSTRACT

The influence of nitrate supplementation and ultraviolet-B (UVB; 280–320 nm) enhancement was tested on a coastal phytoplankton assemblage from eastern Canada exposed to ambient or supplemental UVB irradiance, equivalent to a local 60% ozone depletion. During a 10 d-long mesocosm experiment, 24-h surface bag incubations were repeated three times with half the bags supplemented with nitrate, phosphate and silicate while the other half received only phosphate and silicate. At beginning and the end of these 24-h surface incubations, chlorophyll fluorescence measurements were performed and the abundance of photoprotective pigments diadinoxanthin and diatoxanhtin, of the PSII reaction center D1 protein and of the large subunit of the ribulose-1,5-biphosphate carboxylase/oxygenase enzyme (RuBisCO LSU) was determined. Results showed that as long as nutrients were abundant inside the mesocosms, phytoplankton exposed to supplemental UVB at the surface were able to maintain their maximal quantum yield of PSII fluorescence. As nutrients became limiting inside the mesosocoms, however, phytoplankton showed an increased sensitivity to supplemental UVB and suffered more net photodamage to the PSII reaction centers (seen as decreases in D1 protein abundance). Supplemental UVB also resulted in low abundance of RuBisCO LSU and exacerbated photoinhibition compared to the phytoplankton exposed to surface ambient irradiance. Supplementing nitrate during this nutrient deficient period limited the inactivation of the PSII reaction centers and lowered photoinhibition. Nitrate supplementation had no clear effect on the abundance of the D1 protein but it helped the community to maintain a greater abundance of RuBisCO LSU. Overall, results from this study suggest that the sensitivity of the RuBisCO enzyme to the combined effects of supplemental UVB and nitrate limitation can influence the tolerance of PSII to UVB stress.

1. Introduction

Aquatic primary producers depend on light to energize photosynthetic nutrient assimilation and carbon fixation. Their requirement for solar radiation renders them vulnerable to ultraviolet radiation (UV; 280–400 nm). Cellular proteins are especially sensitive because they absorb in the UV region of the solar spectrum (reviewed in Vincent and Neale, 2000). Ultraviolet-B (UVB; 280–320 nm) can influence protein synthesis through inhibition of macromolecular synthesis (Nishiyama et al., 2006), or by inhibition of nitrogen (N) uptake and metabolism (Döhler, 1997, 1998). At the level of the photosynthetic apparatus, photosystem II (PSII) is a primary UVB target (Aro et al., 1993) while photosystem I (PSI) is relatively insensitive to UVB damage (Strid et al., 1990). In PSII, several possible sites of damage are associated with the D1 protein (reviewed by Franklin et al., 2003), one of the core proteins involved in a PSII repair cycle (Aro et al., 1993). The primary enzyme involved in CO_2 fixation, ribulose-1,5-bisphosphatase carboxylase-oxygenase (RuBisCO) is also a suspected target of UVB inhibition (Strid et al., 1990; Wilson et al., 1995; Lesser, 1996). Both PSII and RuBisCO have been shown to be affected by UVB during the same experiments (Lesser, 1996; Lesser et al., 1996).

N limitation in marine environments (Dugdale, 1967) also profoundly affects cellular proteins, the largest cellular nitrogenous pool. In their study examining the effects of N limitation on the photosynthetic efficiency of PSI and PSII in diatoms and cyanobacteria, Berges et al. (1996) showed that major N limitation effects appeared mainly at PSII rather than at PSI so that diatom species, with abundant PSII centres, were more affected by N limitation than were cyanobacteria with a higher relative PSI content. Dramatic decreases in maximal quantum yields of PSII fluorescence (F_v/F_m) accompanied by declines in D1 protein content were observed in the N-limited cyanobacterium *Prochlorococcus marinus* (Steglich et al., 2001) and in the N-starved diatom *Phaeodactylum tricornutum* (Geider et al., 1993).

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Similarly, declines in F_v/F_m accompanied by a slow decrease in cell chlorophyll (Chl) and protein content were also reported for the N-starved green alga *Dunaliella tertiolecta* (Young and Beardall, 2003), with N resupply stimulating rapid and complete recovery of F_v/F_m within 24 h. Bergmann et al. (2002) found that N-limited phytoplankton from the Neuse Estuary were not more susceptible than N-replenished phytoplankton to photoinduced impairment and downregulation of PSII but that the repair capacity of N-limited phytoplankton was adversely affected. Declines in RuBisCO large subunit (LSU) abundance have also been observed in the nutrient-limited green alga *D. tertiolecta* (Geider et al., 1998) while in the N-starved diatom *P. tricornutum*, a decline in the abundance of the small subunit of RuBisCO (SSU) was observed (Geider et al., 1993).

In the field, phytoplankton are continuously submitted to a combination of varying light and nutrient conditions. Some studies report no marked effects of UVB on the growth of a diatom under N limitation (Behrenfeld et al., 1994) while others report an increased sensitivity of photosynthesis to UVB for N-limited cultures of the diatom *Thalassiosira pseudonana* (Lesser et al., 1994) and of estuarine dinoflagellates (Litchman et al., 2002). In both these studies, the increased sensitivity to UVB in the N-limited cultures was likely the consequence of less efficient repair of UVB damage. In fact, Shelly et al. (2002) found increased inhibition of photosynthesis in the green alga *D. tertiolecta* when UVB was coupled with N limitation but also observed a stimulation of the repair rates which, nevertheless, were not fast enough to counteract the increase in damage. Experiments performed on natural phytoplankton communities have also generated conflicting results. In their study on phytoplankton from a system typical of the upper Great Lakes (Colpoys Bay), Furgal and Smith (1997) found that the varying nutrient status and the photoacclimation state of phytoplankton had no significant influence on their sensitivity to UVB (in terms of photosynthesis-irradiance responses) and that the midsummer mixed community of diatoms, chrysophyceans and green algae was as sensitive as spring diatoms or fall communities. In contrast, Wulff et al. (2000) found that nutrient (nitrate, phosphate, and silicate) supplementation reduced the effects of UVB on Chl *a*, algal intracellular storage of carbohydrates and concentration of extracellular "colloidal" carbohydrates in a diatom-dominated marine microphytobenthic community growing on a sand-substratum. Hence, the responses of phytoplankton to combined UVB and nutrient stress can not be summarised simply. Indeed, it is essential to consider factors such as the duration of the experiments, the extent of phytoplankton exposure to UVB, which is itself influenced by the presence or not of mixing, and the degree of nutrient limitation (Vernet, 2000). The gradual induction of photoprotective screening mechanisms such as the synthesis of mycosporine-like amino acids (MAAs) and the accumulation of carotenoids, detoxifying enzymes or antioxidants must also be considered because they can modulate phytoplankton responses to UV exposure (Vernet, 2000).

Mesocosm experiments can be useful to follow the UVB response of natural phytoplankton communities in a natural setting. Longhi et al. (2006) used a combination of long-term (10 days) mesocosms and short-term, 24 h surface incubations to look at the combined effects of enhanced UVB and nitrate supplementation on taxon-marker pigments and maximal quantum yield of PSII fluorescence from two temperate (Canada, Argentina) and one tropical (Brazil) phytoplankton assemblages. At the temperate Canadian site, a substantial diatom bloom took place and declined over the duration of the experiment. Supplemental UVB generally reduced the growth of diatoms and caused photoinhibition of photosynthesis. Nitrate supplementation in the surface bags slightly relieved photoinhibition but only when nutrients in the mesocosms became limiting to phytoplankton growth (Longhi et al., 2006).

To examine in more details how the photosynthetic system responded to the UVB and nitrate treatments, chlorophyll fluorescence measurements were performed and the abundance of the D1 protein and of RuBisCO LSU determined in the same experiment. As diatoms possess abundant PSII centres and since N limitation affects mostly PSII (Berges et al., 1996), we predicted a stronger influence of additional UVB and nitrate on both D1 and the fraction of active PSII reaction centers during the N-limited bloom decline phase.

2. Materials and methods

2.1. Study site and experimental set-up for the mixed mesocosms

The mesocosm experiment was conducted in eastern Canada (Rimouski; 48.4 °N, 68.5 °W) from 18 to 27 June 2000. The experimental set-up has been described in detail elsewhere (Bouchard et al., 2005). Briefly, six mesocosms (~1800 l) made of transparent polyethylene bags transmitting 85% to 93% in the 280 to 750 nm range, were immersed in the water column and tied to a wharf. These mesocosms were filled simultaneously with 500 µm filtered local coastal seawater one day prior to the beginning of the experiments. Water was collected at 5 m from the surface in the St. Lawrence Estuary. Water inside the mesocosms was mixed using a Little Giant® pump (model 2-MD-HC, Little Giant Pump Co., USA) with a mixing time of ~1 h. Three mesocosms were exposed to natural ambient irradiance (NUVB) and used as controls while the three other mesocosms were exposed to ambient irradiance with additional UVB from lamps resulting in high UVB conditions (HUVB). UVB enhancements simulating a local 60% ozone depletion scenario (Díaz et al., 2006) were obtained using four UVB fluorescent light tubes (Philips TL40 W/12RS, Canada) suspended at ~35 cm above the water surface of the mesocosms. These light tubes, which were preburned for >100 h prior to the experiment and covered with cellulose diacetate sheets changed daily to filter out ultraviolet-C radiation, were turned on from 10:00 am to 3:00 pm using an electronic dimming ballast (Díaz et al., 2006). Wooden dummies were installed on top of the NUVB mesocosm to simulate shading of PAR caused by the lamps over the HUVB mesocosms.

2.2. Short-term surface incubations with nutrient supplementation

During a 10-d period, the progression of nutrient limitation and phytoplankton growth was followed inside the six mixed mesocosms described above. At 6:00 pm on days 1, 5 and 9 of the 10-d experiment (corresponding to the pre-bloom, bloom and post-bloom phases), surface short-term incubations (lasting 24 h) were started using water from two (one NUVB and one HUVB) of the six mixed mesocosms. For each of these surface incubations, six 4 l UV-transparent Ziploc-type bags (transmitting 86% to 94% of light in the 280 nm to 750 nm range) were filled with water originating from the NUVB mesocosm while six other bags were filled with water originating from the HUVB mesocosm. Half of these bags were supplemented with nitrate, phosphate and silicate ("NPS-bags") while the other half was only supplemented with phosphate and silicate and acted as controls ("PS-bags"). Initial nutrient concentrations in the small incubation bags were chosen as a function of natural nutrient concentrations measured below the mixed layer at the experimental site, to mimic mixing events with deeper waters. The nutrient concentrations added were 15 µM nitrate, 1 µM phosphate and 15 µM silicate. Samples for laboratory analyses were collected at the beginning (T_0) and after 24 h of surface incubation (T_F) for each of the three surface incubations performed. The variables examined included nutrient concentrations, Chl *a* biomass, chlorophyll fluorescence-based measurements, and the abundance of photoprotective pigments and photosynthetic proteins.

2.3. Radiometric and temperature measurements

Incident solar radiation was recorded for wavelengths 305, 313, 320, 340, 380 nm and PAR every 10 min using a GUV541 surface

radiometer (Biospherical Instruments, Inc., USA) set on the wharf next to the mesocosms. Irradiance at the surface of the mesocosms was obtained from incident irradiances corrected for the ratio $I_{surface}/I_{incident}$ (shading effects from lamps/dummies). Daily fluence was calculated by integrating these values from 0 to 24 h each day. The daily fluence averaged over the whole water column of the mesocosms was calculated using Riley's equation (Riley, 1957) by multiplying daily fluence at surface by $(1 - e^{-K_d.Z})$ and dividing by $(K_d.Z)$ where K_d refers to the vertical attenuation coefficient and Z refers to the depth of the mesocosms. To assess the relative irradiance increase provided by the UVB lamps, surface irradiances at the five GUV wavelengths from a sunny day around noon in NUVB and HUVB mesocosms were used to integrate over the whole UV range. The average diatoms and dinoflagellates biological weighting function of Neale and Kieber (2000) was then used to assess the weighted lamp-induced UV increase (all UV wavelengths were considered as the lamps caused a slight UVA increase even though their peak is at 313 nm). A PUV-542T radiometer (Biospherical Instruments, Inc.) was used for vertical profiles (same wavelengths as above), enabling the determination of the vertical attenuation coefficient (K_d, m^{-1}) and mean depth of 1% light penetration ($Z_{1\%}$, m). Surface water temperature was continuously measured using thermocouples (type "J") connected to a data logger (21X, Campbell Scientific, Inc., Canada).

2.4. Nitrate and pigment determination

For nitrate measurements, samples were filtered onto precombusted Whatman® GF/F filters and the filtrates were then frozen at −80 °C for later determination of nitrate concentration according to Parsons et al. (1984) using a Technicon II™ autoanalyser. For pigment determination, samples were filtered on Whatman® GF/F filters which were frozen in liquid N_2 and then conserved at −80 °C prior to analyses which were done using high-performance liquid chromatography (HPLC) according to Wright et al. (1991) as modified by Roy et al. (1996). Water samples used for photoprotective pigments (diadinoxanthin, Dd, and diatoxanthin, Dt) analyses were collected at T_0 and at T_F of the surface incubations. Xanthophyll cycling of these two carotenoids involves the conversion of mono-epoxide Dd into the de-epoxide form Dt under high light and, the conversion of Dt back into Dd under low light or darkness (Casper-Lindley and Bjorkman, 1998). The time scales for the conversion of Dd to Dt is very fast and has been reported to vary from minutes to hours (Demers et al., 1991; Olaizola and Yamamoto, 1994). In our sampling design, samples were collected and kept in opaque containers for ~1 h, supposedly long enough for conversion of Dt back into Dd. Despite of this, Dt was still detected in our samples and this coincides with the retention of Dt observed under certain circumstances (Demmig-Adams et al., 1999). The long-term Dt retention observed in our study was expressed as Dt/Dd+Dt and the ratio of (Dd+Dt)/Chl *a*, or pool size of photoprotective pigments, was used to indicate a photoadaptive response to high light intensities (Brunet et al., 1993).

2.5. Chlorophyll fluorescence measurements

Chl *a* fluorescence was measured using a multiple turnover Xenon-Pulse Amplitude Modulated Fluorometer (Xe-PAM, Walz, Germany). Prior to analysis, phytoplankton samples were kept at *in situ* temperature and were dark adapted for at least 45 min to allow for relaxation of non-photochemical quenching (NPQ). To determine the maximal quantum yield of PSII fluorescence, F_v/F_m, minimal fluorescence (F_o) was determined on the dark-adapted sample then a 600 ms saturation pulse of approximately 4000 µmol photons m^{-2} s^{-1} emitted by a Schott saturation flash lamp (Schott Inc., Germany) was administered in order to measure the maximum fluorescence (F_m). These values were used to estimate F_v/F_m with $F_v=(F_m - F_o)$. To determine the steady-state fluorescence parameters, the samples were irradiated with an actinic light set at an intensity similar to that prevailing at the water surface at the time of sampling (T_0 and T_F). F_s, the steady state of fluorescence under actinic light, was determined immediately prior to the saturating pulse and the maximal (F_m') fluorescence was determined following a 600 ms saturation pulse. The sample was then darkened for a short period of 20 s and the minimal fluorescence (F_o') then determined. Using these parameters, the effective quantum yield of PSII fluorescence ($\Delta F/F_m'$) was determined with $\Delta F=F_m' - F_s$ (Genty et al., 1989). The photochemical fluorescence quenching (q_P), the relative reduction state of Q_A, the primary quinone receptor of PSII ($1-q_P$; Havaux et al., 1991), and the non-photochemical quenching (NPQ) were calculated according to Maxwell and Johnson (2000):

$$q_P = (F_m' - F_s)/(F_m' - F_o')$$

$$1 - q_P = 1 - [(F_m' - F_s)/(F_m' - F_o')]$$

$$NPQ = (F_m - F_m')/F_m'$$

2.6. Protein detection by immunoblotting

Total protein, extracted from equivalent original volumes of seawater, was loaded in gel lanes and separated by electrophoresis on linear 10% SDS-polyacrylamide ReadyGels (BioRad, Canada) at 100 V for 100 min in 25 mM Tris-base, 192 mM glycine and 0.1% SDS. After electrophoresis, the separated proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane (BioRad, Canada) in 49 mM Tris-base, 39 mM glycine, 0.04% SDS and 20% methanol in a Semi-Dry Transfer Cell (BioRad, Canada) at 13 V for 25 min. The PVDF membrane was then washed in Tris-buffered saline (TBS-T: 20 mM Tris-base, 500 mM NaCl, 0.05% Tween-20; pH 7.5) and soaked for 1 h at 25 °C in 5% low-fat powdered milk in TBS-T. The primary antibodies used in the immunodetections were Global Antibodies (AgriSera, Sweden, http://www.agrisera.se) raised in chickens against conserved peptide sequence tags diagnostic of the target protein family (PsbA, RbcL) and used according to the supplier recommendations. Primary anti-PsbA global antibody and anti-RbcL antibody were incubated with the membrane in a 1:4000 (PsbA) and1:8000 dilution (RbcL) with blocking buffer (5% low fat milk powder in TBS-T) for 40 min, at 25 °C. A secondary rabbit antichicken IgG conjugated to horseradish peroxidase (Sigma-Aldrich, St Louis, MO, USA) was incubated with the membrane in a 1:4000 dilution with blocking buffer for 40 min, at 25 °C. Between all steps, the PVDF membrane was washed with TBS-T. The proteins were visualized with the ECL+ Plus (Amersham Biosciences, USA) chemiluminescence detection system according to the manufacturer's protocol. The chemiluminescence reaction was imaged with a Fluor-S-Max MultiImager (Bio-Rad, Laboratories Ltd., Canada) using the Quantity One software package (BioRad, Canada). Relative quantities of the detected proteins were calculated using the Quantity One® software package based on the integrated intensity of the chemiluminescence reaction. The relative protein density (pixel count mm^{-2}) for each band was then expressed per l of seawater and subsequently standardized per Chl biomass (µg Chl *a* l^{-1}).

2.7. Statistical analyses

For Chl *a*, chlorophyll fluorescence measurements and pigment data, repeated measures two-way ANOVAs (SYSTAT, V. 9.0, SPSS Inc., USA) were performed with light (UV) and nutrient (Nut) treatments acting as factors and incubation day (days) acting as the repeated factor. When significant differences were present ($p<0.05$), a two-way ANOVA was performed for each specific incubation time (at T_0 and T_F of each surface incubation) with light and nutrient treatments acting as factors followed by a Tukey test whenever a significant effect was

Table 1
Results for the light data

Incubation	1	2	3
Noon incident irradiance			
PAR (µmol photons/m^2/s)	2070	2098	2000
UVA (340 nm) (µW/cm^2/nm)	59.1	58.3	54.6
UVB (305 nm) (µW/cm^2/nm)	4.6	4.6	4.2
Daily integrated surface irradiance			
PAR (mol photons/m^2)	41.61	38.73	29.75
UVA (340 nm) (kJ/m^2/nm)	7.92	7.61	5.67
305 nm NUVB (kJ/m^2/nm)	0.43	0.41	0.29
305 nm HUVB (kJ/m^2/nm)	5.68	5.72	2.77
Daily average mesocosm irradiance			
PAR (mol photons/m^2)	19.02	14.09	10.10
UVA (340 nm) (kJ/m^2/nm)	1.04	1.00	0.65
305 nm NUVB (kJ/m^2/nm)	0.04	0.03	0.02
305 nm HUVB (kJ/m^2/nm)	0.53	0.43	0.18
$Z_{1\%}$			
PAR (m)	5.76	4.19	3.84
UVA (340 nm) (m)	1.40	1.40	1.21
UVB (305 nm) (m)	1.05	0.85	0.78
Ratio all UV HUVB/NUVB			
Unweighted	1.73		
Weighted for biological activity	3.92		

Incident irradiance at noon time (10 min averages) for PAR, UVA (340 nm) and UVB (305 nm) for the three surface incubations. Daily fluence at water surface in the mesocosms (integrated over the incubation period i.e., from 6:00 pm the first day of the surface incubations to 6:00 pm the following day) for PAR, UVA and UVB for both light treatments. Daily fluence averaged over the mesocosm depth (2.3 m) for PAR, UVA and UVB. Mean depth of 1% light penetration ($Z_{1\%}$) for PAR, UVA and UVB. The relative UV irradiance increase (unweighted and weighted for biological activity (Neale and Kieber, 2000) provided by the lamps (relative to NUVB) just below water surface at noon on a sunny day.

observed. Prior to the repeated measures ANOVAs, normality was checked using a one-sample Kolmogorov-Smirnov test whereas the sphericity assumption that concerns variance homogeneity was checked using the Huynh-Feldt epsilon. Only protein determinations originating from the same electrophoresed gel could be compared between them. Because of the limited number of wells on a gel, each gel was loaded with samples taken under both nutrient conditions under one light treatment. Student's *t*-test for independent samples was thus used to compare protein abundance of the two nutrient treatments under one light treatment (PS-bags compared to NPS-bags for either NUVB or HUVB; NUVB and HUVB samples were loaded on different gels).

3. Results

3.1. Light conditions, UVB enhancement and water temperature in the mesocosms

For the days when surface incubations were performed, the average noontime maximal incident irradiance were ~4.5 µW cm^{-2} nm^{-1} for UVB (305 nm), ~57.3 µW cm^{-2} nm^{-1} for UVA (340 nm) and ~2056 µmol photons m^{-2} s^{-1} for PAR (Table 1). Increases in relative UV irradiance, provided by the lamps, were 3.92 fold above ambient when weighted for biological activity (Neale and Kieber, 2000) (unweighted increase = 1.73 fold) (Table 1). This was measured at noon on a sunny day, just below the water surface. Water temperature at the surface of the mesocosms was ~11 °C.

3.2. Nitrate and chlorophyll a concentrations in the mesocosms

Initial nitrate concentrations inside the mixed mesocosms were high (15 µM) while Chl *a* concentrations were less than 1 µg Chl *a* l^{-1} (Fig. 1). With time, Chl *a* concentrations increased to reach near 25 µg Chl *a* l^{-}1 on day 7 and generally declined thereafter. On day 9 only, Chl *a* concentration was significantly greater under supplemental UVB compared to ambient UVB (Fig. 1). Nitrate concentrations continuously decreased as it was being used for phytoplankton growth (Fig. 1). During this experiment, the phytoplankton bloom was dominated by diatoms (*Thalassiosira* and *Chaetoceros* sp.) but phytoflagellates were also present in these mesocosms at fairly steady absolute counts,

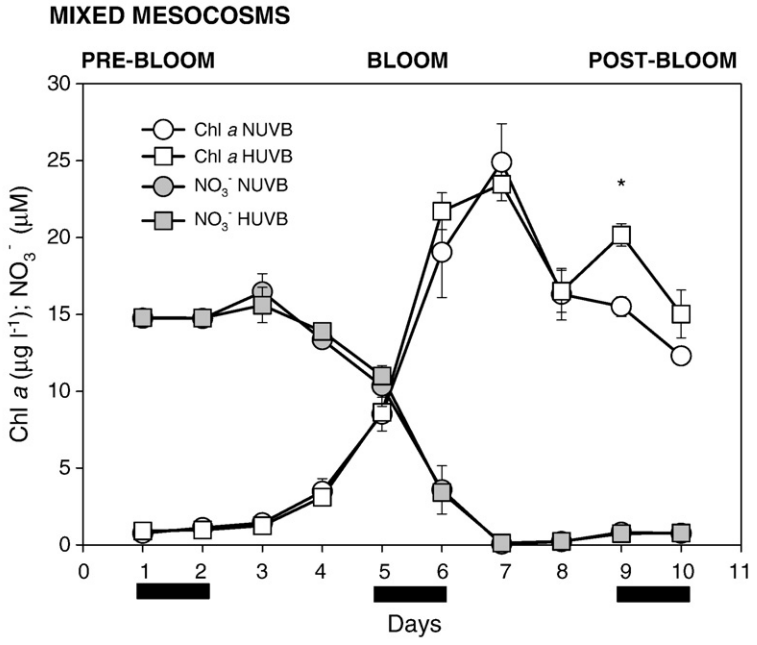

Fig. 1. Nitrate and Chl *a* concentrations inside the mixed mesocosms during the 10-d experiment. Treatments were ambient light (NUVB) and ambient light with lamp-supplemented UVB (HUVB). Periods when surface bag incubations were conducted are indicated by the black rectangles. The first surface bag incubation was performed during the pre-bloom period (from day 1 to day 2), the second during the phytoplankton bloom period (from day 5 to day 6) and the third during the post-bloom period (from day 9 to day 10). Data are the averages±SE of 3 replicate mesocosms. *=significant ($p \leq 0.05$) UVB effect.

although their relative abundance varied through time as the diatom bloom progressed (Ferreyra et al., 2006; Roy et al., 2006). The phytoflagellate group was mainly composed of chlorophytes, euglenophytes, prasinophytes and small (<5 μm) unidentified phytoflagellates (Ferreyra et al., 2006; Roy et al., 2006).

3.3. Timing of the surface incubations

Surface incubations were performed during the phytoplankton pre-bloom, bloom and post-bloom periods, respectively (Fig. 1). The POC:PON molar ratio was ~8.5 and 6.5 at the beginning of the first and the second surface incubations, respectively, while it was near 20.0 at the beginning of the third surface incubation (Longhi et al., 2006), which is consistent with the bloom development and its decline towards the end of the 10-d experiment.

3.4. Chlorophyll a concentrations during surface incubations

Nitrate supplementation at the beginning of each of the three surface incubations had no clear effect on the Chl *a* during the subsequent 24 h incubation period (Fig. 2A–B). Initial Chl *a* concentrations were and remained lower than 1 μg Chl *a* l^{-1} under all conditions tested during the course of the first surface incubation but increased from near 7 μg Chl *a* l^{-1} to more than 10 μg Chl *a* l^{-1} under all conditions tested during the course of the second surface incubation (Fig. 2B). This increase in Chl *a* concentrations was also observed under all conditions tested during the course of the last surface incubation. No significant differences were observed between the different treatments tested (Fig. 2B, Table 2).

Table 2
Summary of results obtained from the repeated-measures ANOVA for Chl a, chloroplhyll fluorescence measurements and photoprotective pigments during surface incubations

	UV	Nut	UV×Nut	Days	Days×UV	Days×Nut	Days×UV×Nut
Chl *a*	ns	ns	ns	***	ns	ns	ns
F_v/F_m	*	*	ns	***	ns	ns	ns
$\Delta F/F_m'$	*	ns	ns	***	ns	ns	ns
$1 - q_P$	ns	*	ns	***	*	ns	ns
NPQ	ns	**	ns	***	ns	***	ns
Dd+Dt/Chl *a*	ns	ns	ns	***	*	ns	ns
Dt/Dd+Dt	ns	ns	ns	***	***	ns	ns

The source of variation is partitioned into individual effects of the light (UV) and nutrient treatment (Nut), their interaction (UV×Nut), the repeated measure (Days) and its interaction with the individual effects (Days×UV, Days×Nut, Days×UV×Nut). * Indicates significance at $0.01 < p \leq 0.05$, ** $0.001 < p \leq 0.01$, *** $p \leq 0.001$, ns: not significant.

3.5. Chlorophyll fluorescence measurements during surface incubations

Phytoplankton were in a poor physiological state ($F_v/F_m < 0.2$) when the first surface incubation was performed (pre-bloom) but this improved over the course of the mesocosm experiment ($F_v/F_m > 0.45$ at T_0 of the second and third surface incubations; Fig. 3). Exposing the phytoplankton to surface ambient irradiance for 24 h at this site generally caused a photoinhibitory decline in F_v/F_m (Fig. 3). During the second and third surface incubations, supplemental UVB caused more photoinhibition, as seen from significantly larger declines in F_v/F_m under HUVB than under NUVB. During the third surface incubation (post-bloom) nitrate supplementation significantly alleviated F_v/F_m declines under both light treatments (Fig. 3).

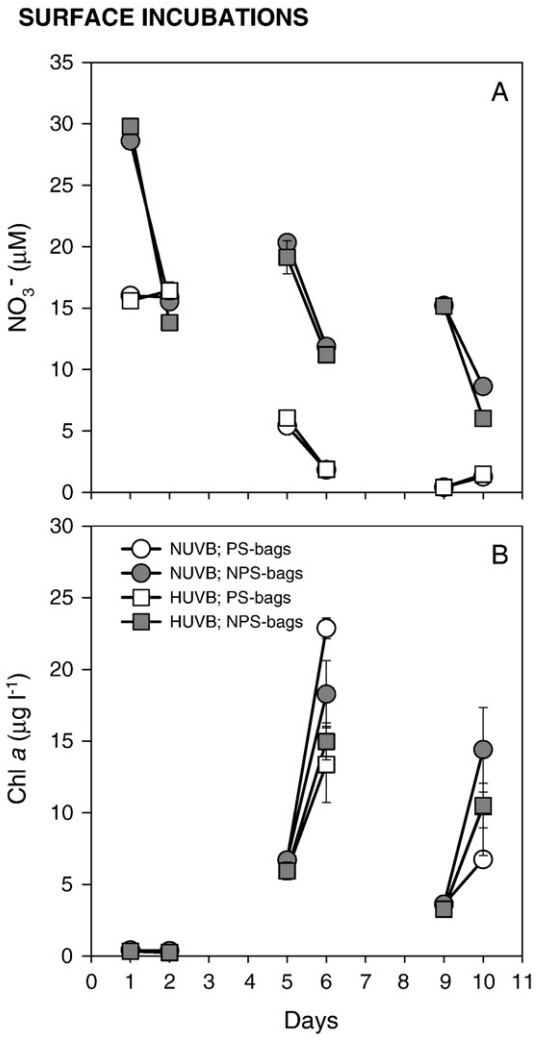

Fig. 2. Nitrate (A) and Chl *a* (B) concentrations determined at T_0 and T_F of the surface incubations. Treatments were ambient light (NUVB) and ambient light with lamp-supplemented UVB (HUVB), with the addition of nitrate, phosphate and silicate (NPS-bags) or the addition of phosphate and silicate only (PS-bags). Data are the averages±SE of 3 replicate bags.

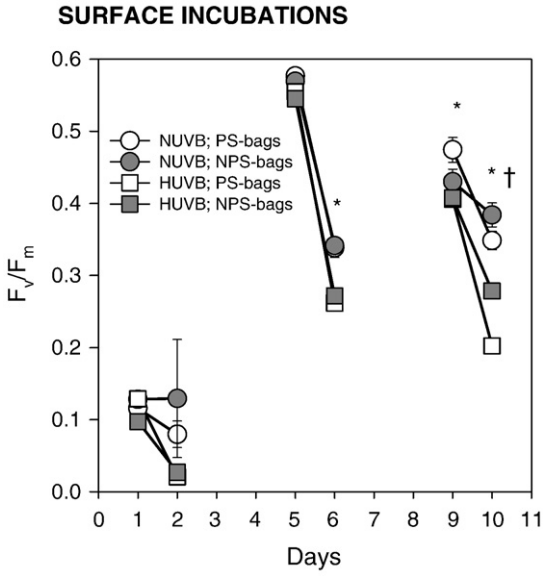

Fig. 3. Maximal quantum yield of PSII fluorescence (F_v/F_m) determined at T_0 and T_F of the surface incubations. Treatments are as described in Fig. 2. Data are the averages±SE of 3 replicate bags. *=significant ($p \leq 0.05$) UVB effect; †=significant ($p \leq 0.05$) nutrient effect.

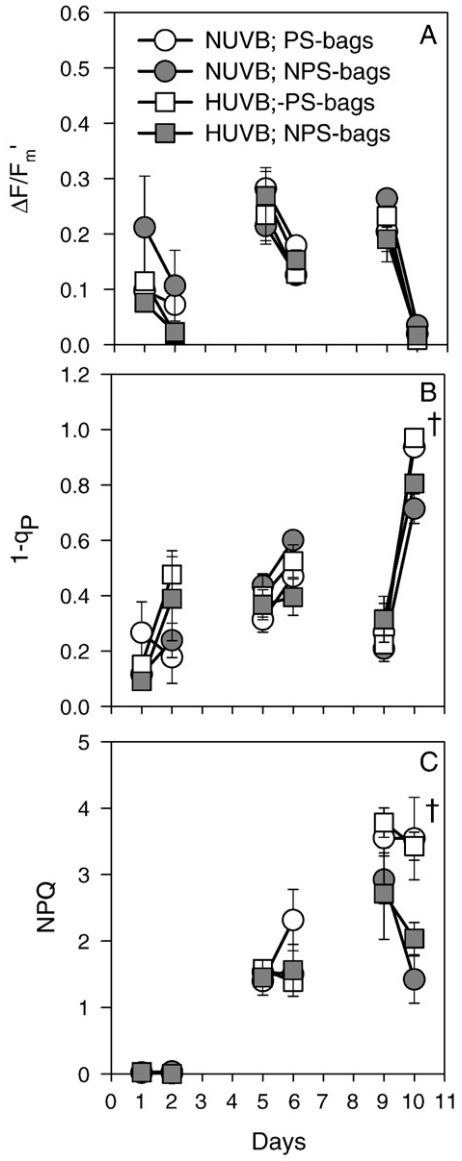

Fig. 4. Effective quantum yield of PSII fluorescence ($\Delta F/F_m'$; A), fraction of inactive PSII reaction centers ($1 - q_P$; B), and non-photochemical quenching (NPQ; C) determined at T_0 and T_F of the surface incubations. Treatments are as described in Fig. 2. Data are the averages±SE of 3 replicate bags. †=significant ($p \leq 0.05$) nutrient effect.

The effective quantum yield of PSII fluorescence ($\Delta F/F_m'$) remained relatively low (<0.3) throughout the course of the three surface incubations and decreased under all conditions tested during the 24 h surface incubations (Fig. 4A). In contrast to F_v/F_m, $\Delta F/F_m'$ did not benefit from nitrate supplementation. $1 - q_P$, which relates to the fraction of closed or inactive PSII reaction centers (Havaux et al., 1991), was always lower at the beginning of the surface incubations and generally increased over the 24 h incubation period (Fig. 4B). During the third surface incubation, nitrate supplementation helped the community to maintain a greater fraction of active PSII reaction centers under both light treatments tested ($1 - q_P$ lower in the NPS-bags, Fig. 4B). NPQ, which relates to the dissipation of excess light energy as heat, increased over the course of the mesocosm experiment (T_0 of surface incubations on days 1, 5 and 9; Fig. 4C) but showed similar patterns under both light treatments tested. Over the course of the surface incubations, supplemental UVB had no clear effects on NPQ compared to the ambient light treatment but, during the third surface incubation, nitrate supplementation significantly reduced NPQ in the NPS-bags compared to the PS-bags (Fig. 4C, Table 2).

3.6. Photoprotective pigments during surface incubations

Similarly to NPQ, the Chl-specific pool of photoprotective pigments (Dd+Dt) increased over the course of the experiment (T_0 of surface incubations on days 1, 5 and 9) irrespective of the light treatments (Fig. 5A). The retention of Dt was observed after 24 h of surface incubation under all conditions tested during the second and third surface incubations (Fig. 5B). Nitrate supplementation during the third surface incubation lowered, although not significantly (Table 2), the amount of Dt retained at the end of the 24 h incubation period (Fig. 5B). The relationship between the amount of Dt retained and the fraction of inactive PSII reaction centers showed that the retention of Dt increased in a linear manner in response to $1 - q_P$ (Fig. 5C).

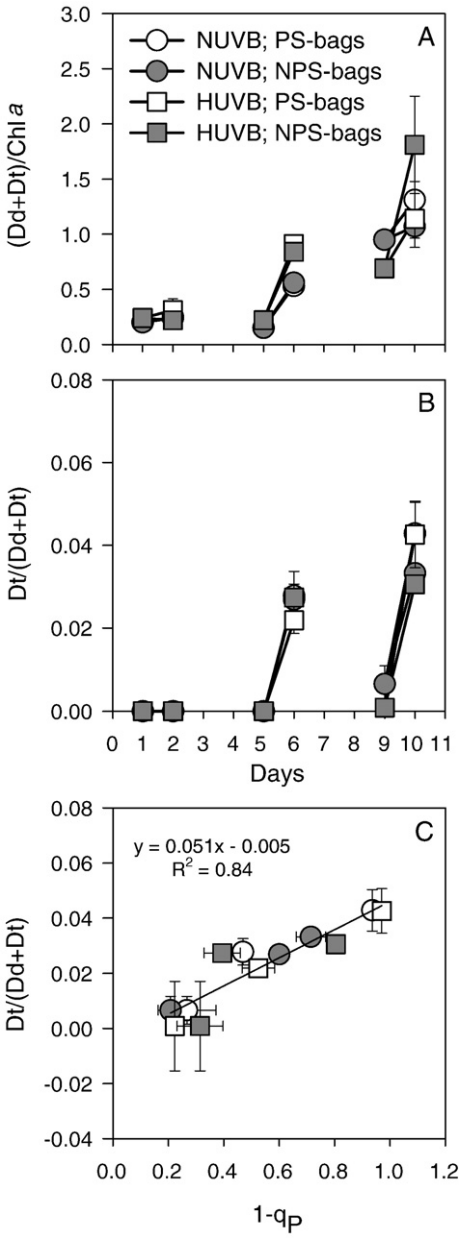

Fig. 5. Chl-specific pool of diadinoxanhin (Dd) and diatoxanthin (Dt) (A), retention of Dt (Dt/Dd+Dt) (B) determined at T_0 and T_F of the surface incubations. Treatments are as described in Fig. 2. Data are the averages±SE of 3 replicate bags. Relationship between Dt retained (Dt/(Dd+Dt)) when Dt retention occurred and values were >0 even after 60 min of darkness and the fraction of inactive PSII reaction centers, $1 - q_P$ (C).

3.7. Photosynthetic proteins during surface incubations

During the course of the first surface incubation, the abundance of the RuBisCO LSU (Fig. 6A) and of the D1 protein (Fig. 6B) increased in 24 h under supplemental UVB while it remained mostly unchanged under NUVB. During the second surface incubation, the abundance of the RuBisCO LSU (Fig. 6A) remained low irrespective of the light treatments while the abundance of the D1 protein decreased under all conditions tested (Fig. 6B). During the third surface incubation, the abundance of the RuBisCO LSU declined under all the conditions tested, particularly under NUVB. However, the lowest RuBisCO LSU abundance was reached under supplemental UVB (Fig. 6A). In contrast, the abundance of the D1 protein remained low but stable under NUVB. Under supplemental UVB, the abundance of the D1 protein was initially higher than under NUVB but it decreased during the surface incubation (Fig. 6B).The RuBisCO LSU to D1 ratio which provides an indication of resource allocation within the photosynthetic apparatus, remained below 0.30 during the course of the first two surface incubations (Fig. 6C). In contrast, during the third surface incubation, this ratio increased from about 0.5 to values between 1 and 2 for the HUVB treatment, and was around 150 at the start of the NUVB treatment. This high ratio is due to a relatively high RuBisCO LSU abundance (Fig. 6A) and very low D1 protein abundance (Fig. 6B). Although the difference between the PS-bags and NPS-bags under a given light treatment was not significant, nitrate supplementation helped to maintain higher ratios of RuBisCO LSU to D1 under both light treatments tested (Fig. 6C). The effect of nitrate supplementation during the course of the last surface incubation can be attributed to the maintenance of higher RuBisCO LSU abundance for both light treatments tested (Fig. 6A) since the D1 protein abundance remained mostly unaffected by this nutritional input (Fig. 6B).

4. Discussion

The confinement in mesocosms of local seawater rich in nutrients resulted, as anticipated, in the development of a large diatom-dominated bloom which slowly declined toward the end of the experiment as nutrients became limiting. During this 10-d experimental period, a series of three fixed surface incubations were conducted inside these mesocosms in order to assess the joint effect of supplemental UVB and nitrate supplementation on phytoplankton assemblages as the bloom grew and declined. Gradual increases in the pool size of photoprotective pigments and in NPQ for the initial samples taken from the mesocosms suggest that the cells used the xanthophyll cycle to dissipate excess excitation energy (Brunet et al., 1993; Olaizola et al., 1994; Demmig-Adams et al., 1999). Since the increase in photoprotective pigment concentrations occurred to a similar extent in both NUVB and HUVB mesocosms, it was likely related to the gradual nutrient depletion occurring inside the mesocosms (Geider et al., 1993; Latasa, 1995) rather than to UVB exposure (Schofield et al., 1995). Since the surface incubation results varied for the three periods tested, each of them is discussed separately.

4.1. Pre-bloom period: Phytoplankton are insensitive to supplemental UVB and unresponsive to nitrate supplementation

The first surface incubation (starting on day 1) was performed before the development of the phytoplankton bloom inside the mixed mesocosms (pre-bloom period) when cells were in a poor physiological state ($F_v/F_m < 0.20$ at T_0 on day 1). This poor state could be due to the transfer, one day before, of cells from a water collection depth of 5 m in the St. Lawrence Estuary to the shallow mesocosms (2.3 m deep). The low photoprotective pigment concentrations and NPQ values observed during this period suggest that these cells were acclimated to lower irradiances. Phytoplankton exposure to supplemental UVB during the first 24 h surface incubation did not clearly affect the different chlorophyll fluorescence measurements or the photoprotective pigments. Phytoplankton cells were likely too stressed (cf. low F_v/F_m) to respond to the enhanced UVB treatment.

At the molecular level, a marked increase in Chl-specific D1 and RuBisCO LSU abundance occurred in the supplemental UVB treatment. These changes are similar to the rapid rise in D1 protein and in RuBisCO LSU abundance observed upon transferring an epiphytic lichen and its photosymbionts from 5 °C to 16 °C at high light (Mackenzie et al., 2004). While in the lichen case these changes were supported by a rapid increase in the effective quantum yield of PSII fluorescence and suggested a general up-regulation of photosynthesis after the shift in temperature (Mackenzie et al., 2004), in the present study, the macromolecular changes observed were not associated with a change in quantum yield of PSII fluorescence. Despite the high D1 and RuBisCO LSU abundance, the RuBisCO LSU to D1 ratio remained similar to the ratio under ambient UVB, i.e., below 0.30. The very low F_v/F_m values measured during this surface incubation suggest that some of the D1 protein detected was not part of functional PSII centers but rather belonged to intermediates of the PSII repair cycle, as found for cyanobacteria (Burns et al., 2005). Nitrate supplementation during this incubation had no effect simply because N was still plentiful inside the mesocosms (nitrate >15 µM; POC:PON ~8.5).

4.2. Bloom period: Phytoplankton are mostly sensitive to high surface irradiance, slightly sensitive to supplemental UVB and unresponsive to nitrate supplementation

Over time, phytoplankton cells confined inside the shallow mesocosms gradually acclimated to the irradiance conditions and cells attained a better physiological state by day 5 ($F_v/F_m > 0.55$ at T_0). Although the Chl-specific photoprotective pigment pool was still small at the start of the second incubation (near 0.25), NPQ values reached 1.5, an indication of photoprotection (Lavaud et al., 2004). Exposing mixed phytoplankton to surface irradiance for 24 h caused decreases in F_v/F_m, $\Delta F/F_m'$, and an increase in the fraction of inactive PSII reaction centers (higher $1 - q_P$ values), suggesting increased photodamage. Supplementing UVB during this period exacerbated photoinhibition, since F_v/F_m declined, which is consistent with other UV studies in diatoms (Hazzard et al., 1997; Leu et al., 2006). Retention of the de-epoxidated xanthophyll (Dt/(Dd+Dt)) increased during the incubations, but was similar for the two light and nutrient treatments. This retention can explain the decrease observed in $\Delta F/F_m'$ (Adams et al., 1995). The relatively high and mostly unchanged NPQ values observed during the course of this second surface incubation indicate that the principal response of phytoplankton was to the high surface irradiance, not the supplemental UVB level. During this surface incubation, as for the first surface incubation, nitrate supplementation had no effect on the measured variables because N was still abundant inside the mesocosms (nitrate >5 µM; POC:PON ~6.5).

4.3. Post-Bloom period: Phytoplankton are sensitive to supplemental UVB but benefit from nitrate supplementation

On day 9, the first day of the third surface incubation, the initial F_v/F_m value was near 0.45, the pool size of Chl-specific photoprotective pigments was near 1 and NPQ values were greater than 3. This indicates greater photodamage at the start of the incubation (highest starting NPQ of all three series of incubations) associated with an increase in photoprotective pigments (Lavaud et al., 2002). Exposing phytoplankton from the mixed mesocosms to surface irradiance for 24 h during this post-bloom period caused a decrease in F_v/F_m, a decline to near zero of $\Delta F/F_m'$ and a dramatic increase in the fraction of inactive PSII reaction centers (increased excitation pressure). The retention of Dt observed under both light treatments at the end of the

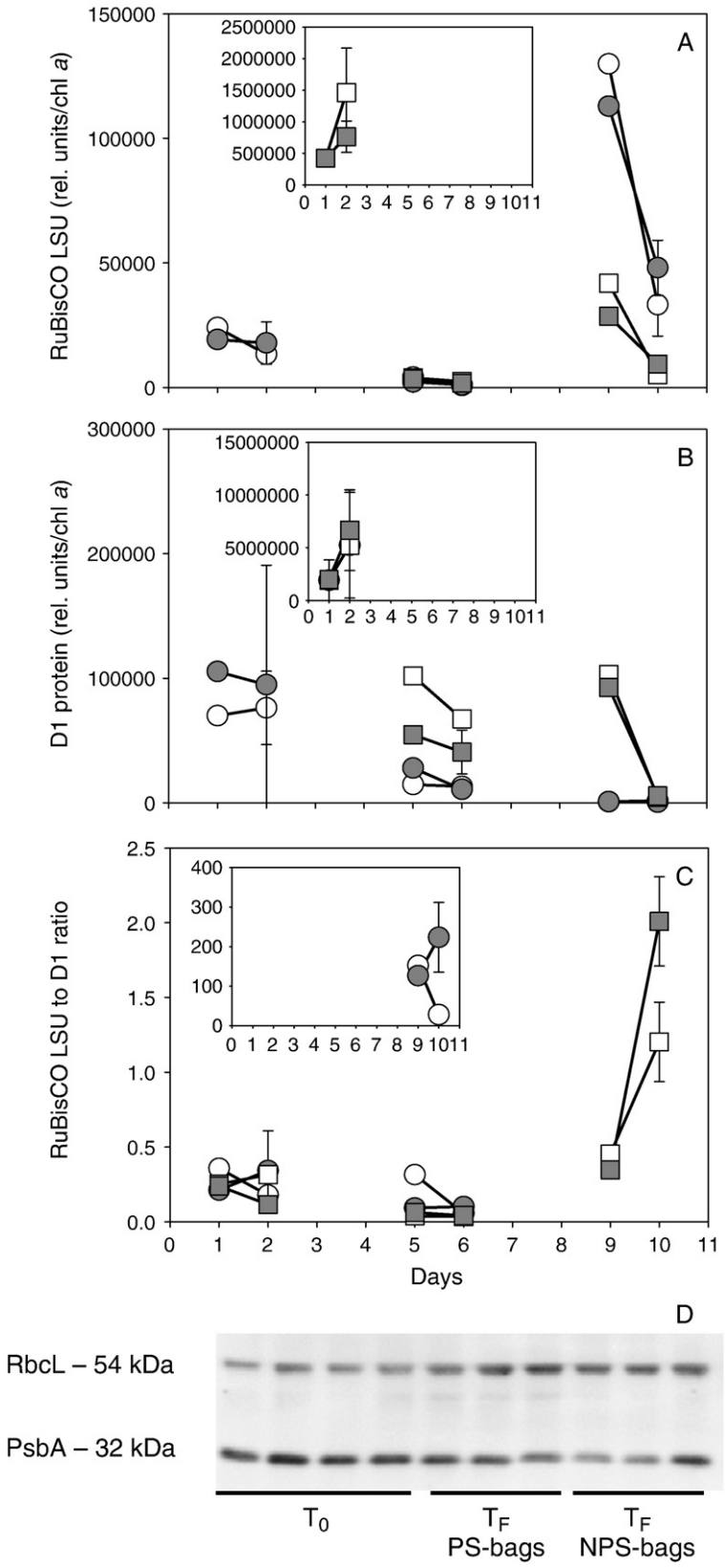

Fig. 6. Chl-specific RuBisCO LSU abundance (A) and Chl-specific D1 protein abundance (B) determined at T_0 and T_F of the surface incubations. Fig. 6 A, B insets: Chl-specific RuBisCO LSU and D1 protein abundance, respectively, determined at T_0 and T_F of the first surface incubation for the HUVB light treatment only. RuBisCO LSU to D1 protein ratio determined at T_0 and T_F of the surface incubations (C). Fig. 6 C inset: RuBisCO LSU to D1 protein ratio determined at T_0 and T_F of the third surface incubation for the NUVB light treatment only. Treatments are as described in Fig. 2. Data are the averages of 2 replicate bags for T_0 and the averages±SE of 3 replicate bags for T_F. Representative immunoblot profile of RbcL (RuBisCO large subunit) and PsbA (D1 protein) for the different bags exposed to supplemental UVB during the second surface incubation (D).

24 h surface incubation is similar to the slow reconversion of zeaxanthin to violaxanthin observed in higher plants exposed to long-term stressors (Demmig-Adams and Adams, 1996) when the synthesis rate of various PSII core components is slowed down (Demmig-Adams et al., 1999). A slower synthesis of PSII core components is reflected in the low D1 protein abundance reached by the end of this surface incubation. The typical rapid reconversion of Dt into Dd (Casper-Lindley and Bjorkman, 1998) did not occur in our study even after more than 60 min of darkness, resulting in retention of Dt. This retention increased with the excitation pressure ($1 - q_P$) which was highest after 24 h surface incubation during the post-bloom period. Hence, this prolonged stress (worse in post-bloom) resulted in inactivation of a large fraction of PSII reaction centers and retention of the de-epoxidated pigment (Adams et al., 1995; Demmig-Adams and Adams, 1996). Dt was likely involved in the dissipation of the excess light energy captured to try to protect the cells (Demmig-Adams and Adams, 2006). This response is consistent with the sustained engagement of zeaxanthin and antheraxanthin-dependent energy dissipation associated with an altered PSII protein composition in an overwintering plant (Verhoeven et al., 1998).

Supplemental UVB during this third surface incubation exacerbated photoinhibition compared to the phytoplankton exposed to ambient irradiance. In addition, the significant decline in abundance of the D1 protein under supplemental UVB compared to low but stable D1 levels under ambient irradiance suggests photodamage. This net Chl-specific D1 loss under supplemental UVB supports a previous study of a temperate phytoplankton assemblage showing that supplemental UVB led to a net decline in D1 protein abundance (Bouchard et al., 2005) likely as a result of slowed protein synthesis (Nishiyama et al., 2006). The abundance of the RuBisCO LSU also declined under both light treatments. Despite the greater decline observed in 24 h under ambient irradiance, the Chl-specific RuBisCO LSU abundance reached at the end of the 24 h surface incubation was lower under supplemental UVB, which suggests UVB damage to this enzyme (Lesser et al., 1996; Bischof et al., 2002). Overall, the above observations all point toward net photodamage to the PSII reaction centers and photoinhibition of photosynthesis. The observed response is similar to the short-term inhibition observed upon exposure of a subtropical diatom to ambient UVB for a short (<24 h) period of time, characterised by a reduction of variable fluorescence, RuBisCO activity and increased destruction of the D1 protein (Hazzard et al., 1997). These results also agree with those of Lesser et al. (2002) showing primary UV effects on PSII, most likely at the D1 protein, followed by more chronic effects on carbon fixation mechanism.

In contrast to the surface incubations performed in the previous periods, a clear synergistic effect of supplemental UVB and nitrate limitation was observed during post-bloom surface incubations, when N was limiting (nitrate <0.76 µM; POC:PON ~20). These results are in agreement with previous studies showing an increased sensitivity to UV in nutrient-limited cells (Cullen and Lesser, 1991; Litchman et al., 2002). Nitrate supplementation during this period slightly relieved photoinhibition and helped maintain a greater fraction of active PSII reaction centers, as seen by lower $1 - q_P$ values in the NPS-bags compared to the PS-bags. The maintenance of greater RuBisCO LSU abundance upon nitrate supplementation while the abundance of the D1 protein remained mostly unaffected can be compared to the acclimation response observed in photoautotrophs submitted to low temperature which consists in increasing their capacity for CO_2 assimilation relative to electron transport by elevating their levels of Calvin cycle enzymes (Huner et al., 1998). By ensuring a more efficient downstream removal of electrons (Huner et al., 1998), the maintenance of greater RuBisCO LSU abundance upon nitrate supplementation favoured the maintenance of a greater fraction of active PSII reaction centers available for excitation capture (significantly lower $1 - q_P$ values in NPS-bags compared to PS-bags), limited the excess excitation pressure on PSII and relieved photoinhibition (significantly greater F_v/F_m). The lower excitation pressure on PSII may ultimately have limited the acidification of the lumen and prevented the establishment of pH-dependent NPQ (Demmig-Adams and Adams, 1996; Niyogi, 1999; Wilson and Huner, 2000), shown by significantly lower NPQ in the nitrate-supplemented bags.

4.4. Conclusions and future considerations

This study shows that nitrate-limited phytoplankton can improve their maximal quantum yields of PSII fluorescence if exposed to supplemental nitrate. Phytoplankton here likely allocated supplemental nitrate for the maintenance of the essential RuBisCO enzyme. This ensured a more efficient downstream removal of electrons, which released pressure on PSII and partly relieved photoinhibition. Results from this study suggest that the sensitivity of the RuBisCO enzyme to the combined effects of nutrient limitation and supplemental UVB influences the tolerance of PSII to UVB stress. In order to better predict the fate of primary producers in the context of important global changes, future studies need to focus on assessing the combined effects of multiple stressors on multiple targets in the photosynthetic apparatus of natural phytoplankton assemblages.

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