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Research Article

Induction of thymine dimers by solar radiation in natural freshwater phytoplankton assemblages in Patagonia, Argentina

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Abstract. Natural phytoplankton assemblages from a freshwater lake in Trelew (Province of Chubut, Patagonia, Argentina) were exposed to natural solar radiation at different depths in a water basin filled with fresh water rich in humic substances. Samples were taken at regular intervals for DNA extraction and subsequent analysis of DNA damage by determining the formation of thymine dimers using an immuno-dot-blot procedure. The use of a colorimetric detection system based on the reaction of alkaline phosphatase with BCIP (5-bromo-4-chloro-3-indolyl phosphate)/NBT (nitro blue tetrazolium) proved unsatisfactory because of uneven and strong background signals. As a consequence the antibodies were stripped and the blots were reprobed successfully, using a chemoluminescence based detection system. Natural solar radiation caused an increase from 3.2 ± 0.9 thymine dimers (T^T) per mega base pair (Mbp) to 50.9 ± 3.8 T^T Mbp^{-1} during the light period in natural phytoplankton exposed at the surface. When the UV-B component of the solar radiation was removed by a 320 nm cut-off filter, no thymine dimers were produced. After prolonged exposure at the surface, some but not total recovery occurred overnight. There was no significant DNA damage in the samples exposed at 85 cm depth (k_d = 3.62 m^{-1} at 305 nm). The results indicate that the impinging UV-B radiation at the study site may be deleterious to the survival of phytoplankton exposed at the water surface and thus significantly alter the nutrient cycling and biomass production.

Key words. DNA damage; freshwater phytoplankton; repair; solar UV radiation.

Introduction

A potential threat to phytoplankton is the continuing depletion of the stratospheric ozone layer, as a result of anthropogenically released atmospheric pollutants such as chlorofluorocarbons (CFCs), chlorocarbons (CCs) and organo-bromides (OBs) and the consequent increase in solar UV-B radiation reaching the Earth's surface (Crutzen, 1992; Kerr and McElroy, 1993; Kirchhoff, 1996). In addition to the Antarctic ozone "hole", ozone depletion has also been reported in the north polar regions (von der Gathen et al., 1995) as well as at Northern and Southern mid-latitudes. In the particular case of Patagonia, an area in close proximity to the Antarctic ozone "hole", ozone depletion associated with the polar

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vortex has been documented up to 38° of latitude during October – November (Orce and Helbling, 1997). Also, air masses with poor ozone concentration have been recorded to detach from the vortex and move over Patagonia (Atkinson et al., 1989; Kirchhoff, 1996).

Like all phototrophic organisms phytoplankton are potentially subject to harmful effects of excessive UV-B radiation, such as DNA damage, inhibition of photosynthesis and growth, and finally cell death. In the clearest oceanic waters UV-B radiation can penetrate several tens of meters, and in Antarctica, UV radiation has been detected to penetrate to a depth of 70 m (Smith et al., 1992). Even in coastal waters, characterized by strong attenuation of UV radiation, near surface irradiances are high enough to cause injurious effects in aquatic organisms (Piazena and Häder, 1994). In freshwater environments, the penetration of UV is dependent on the concentration of dissolved organic matter (DOM)(Osburn and Morris, 2002). Although studies on the impact of UV on freshwater ecosystems are less frequent than those on marine ecosystems, there is growing evidence that freshwater organisms are also stressed by UV-B radiation (Beardall et al., 1997; Villafañe et al., 1999; Sommaruga and Buma, 2000; Helbling et al., 2001). Phytoplankton organisms have developed a number of protective mechanisms to avoid harmful UV radiation. These include the vertical movement within the water column (Häder, 1988), repair of DNA damage (Karentz et al., 1991) and the production of UV-absorbing compounds, mycosporine-like amino acids (MAAs) (Carreto et al., 1990; Dunlap et al., 1995; Helbling et al., 1996; Vernet and Whitehead, 1996; Xiong et al., 1997; Sinha et al., 1998; Klisch et al., 2001).

The two major types of UV-induced DNA lesions are cyclobutane pyrimidine dimers (CPDs), of which thymine dimers (T^T) are most frequent, and 6–4 photoproducts (Friedberg et al., 1995). We tested the hypothesis that DNA of freshwater phytoplankton communities might be affected by natural solar radiation and that effective repair mechanisms might be operating that could be dependent on the UV dose and water depth. Therefore, we investigated the effect of natural solar radiation on the DNA of freshwater phytoplankton, by assessing the formation of thymine dimers.

Materials and methods

Experimental site

The experiments were performed in Playa Unión, located on the Argentinean coast (43°18′ S; 65°03′ W) during February 2000. Solar radiation was measured using an ELDONET (Häder et al., 1999) filter radiometer (Real Time Computer, Möhrendorf, Germany) that has three channels for photosynthetically active radiation (PAR, 400–700 nm); ultraviolet-A radiation (UV-A, 315–400 nm); and ultraviolet-B radiation (UV-B, 280–315 nm) that is permanently installed on the roof of the Estación de Fotobiología Playa Unión (Villafañe et al., 2001).

Test organisms

Water samples were obtained from a eutrophic freshwater lagoon in Trelew, approximately 25 km from the experimental site. The samples were taken at approximately 10 cm depth with a polycarbonate bottle cleaned with 1 N HCl and immediately carried to the laboratory in darkness. There they were kept in dim light until the start of the experiments on the subsequent day. The chlorophyll concentration was estimated from methanolic extracts (Porra et al., 1989).

Exposure to solar radiation

The water samples were supplemented with 5 mM $NaHCO_3$ to provide inorganic carbon for photosynthesis, and subsequently dispensed into small polyethylene bags (containing 50 ml each) that were heat sealed. The bags were prepared from larger UV-transparent polyethylene bags (Ziploc, Melitta Toppits, Minden, Germany) by means of a heat sealer. The bags containing the phytoplankton samples were immersed either at the bottom or lay at the surface of a water basin (3 m diameter, 85 cm depth), and exposed to solar radiation. The basin was filled with tap water that was supplemented with water rich in humic substances to enhance the attenuation of UV-B. The water containing humic substances was obtained from a rain water residue at the experimental site. The transmission spectrum of this water is shown in Figure 1. Two treatments were implemented: samples exposed to full radiation (surface and 85 cm depth) and samples that were covered with a 320 nm cut-off film filter (surface only); in addition, a dark control was used at 85 cm depth.

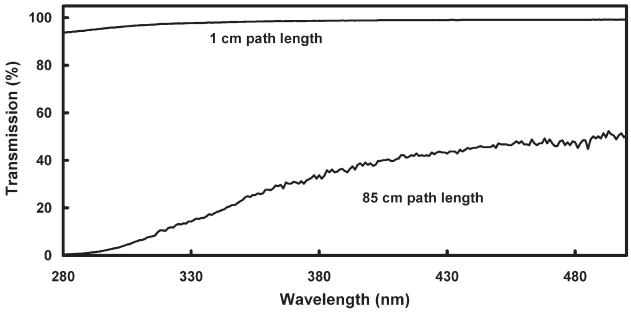

Figure 1. Transmission spectrum of water containing humic substances. Upper curve: transmission at 1 cm optical path length measured spectrophotometrically. Lower curve: transmission at 85 cm optical path length estimated from the spectrophotometric measurement.

Duplicate samples were taken from each treatment, after 4 and 8 h of exposure to solar radiation (light period) and at the end of the subsequent dark period (i.e., 20 h after initiation of the experiments) from the surface and the bottom of the basin. Duplicate samples of the dark controls were also taken after 4 h and after 20 h. Each sample consisted of one polyethylene bag's content. After each sampling, the bag content was quickly transferred to polypropylene tubes, and Lugol's solution was added to a final concentration of 10% to kill the cells and prevent repair processes or degradation of DNA. The samples were allowed to sediment in darkness at 4 °C overnight. Thereafter the supernatants were carefully decanted; the sediments were transferred to 2-ml micro centrifuge tubes and kept in darkness at 4 °C until further analysis in the laboratory at Friedrich-Alexander University (Erlangen, Germany).

DNA extraction and quantification of thymine dimers

The extraction of DNA and quantification of thymine dimers was performed by immuno-dot-blot analysis as described by Sinha et al. (2001) with some modifications. Two different detection systems were used with intermittent stripping of the antibodies and reincubation of the membrane, as the first detection system proved unsatisfactory. The procedure was briefly as follows: The cells were washed three times in TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA) to remove residual Lugol's solution and then sonicated (20 W, Branson Sonifier 450, Ultrasonic Corporation, Danbury, USA) for 3 min on ice to disrupt the cells. The resulting suspension was made up to 1 ml with TE buffer. Aliquots of 20 µg RNAse A (Boehringer, Mannheim, Germany) were added before incubation at 37 °C. After one hour of incubation, 0.05% SDS and 1 mg proteinase K (Boehringer, Mannheim, Germany) were added. After incubation for an additional 8 h the samples were centrifuged and the supernatant extracted once with phenol/chloroform/isoamyl alcohol (25/24/1, v/v) and twice with chloroform/isoamyl alcohol (24/1, v/v). DNA was precipitated by addition of 0.1 volume of 3 M sodium acetate and 2 volumes of 96% ethanol. The precipitate was dried at room temperature and dissolved in 500 µl of TE buffer.

For spectrophotometric determination of DNA concentration and for checking the 260/280 nm absorption ratio, 50-µl aliquots were used. The DNA was transferred to a nylon membrane and incubated with an antibody specific to thymine dimers (Kamiya Biochemicals, Seattle, USA) as described earlier (Sinha et al., 2001). The secondary antibody used was anti-mouse IgG conjugated with alkaline phosphatase. A mixture of BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitro blue tetrazolium) was used as a substrate (SIGMA FAST, SIGMA, Saint Lois, Missouri, USA). The intensity of the color reaction was quantified using a densitometer (Quick Scan Jr. TLC, Desaga, Heidelberg, Germany) and peak areas were determined by planimetry. This procedure using an alkaline phosphatase coupled secondary antibody and BCIP/NBT as a substrate led to an uneven and strong background signal and proved unsatisfactory. As a consequence we decided to strip the antibodies by incubation in 62.5 mM Tris-HCl (ph 6.8), 2% SDS and 100 mM mercaptoethanol at 70 °C for 30 min. Thereafter the assay was repeated as reported (Sinha et al., 2001), using a horseradish peroxidase coupled secondary antibody and a chemoluminescence substrate for detection. The blot was imaged using an imaging station (Kodak Image station 440 CF, Kodak Digital Science, New Haven, CT, USA) and the frequency of thymine dimers calculated as described by Sinha et al. (2001).

Statistics

The unpaired Student's t-test was used for comparisons of DNA damage between experimental groups (n = 2 or 3). A value of $P < 0.05$ was considered statistically significant.

Results

The time course of solar radiation in the PAR, UV-A and UV-B wavebands on the days of the experiments is depicted in Figure 2. 22 February 2000 was a clear day till late afternoon, when some clouds appeared. Peak values of PAR, UV-A and UV-B of 410, 54 and 1.1 W m^{-2}, respectively, were recorded around local noon. 25 February 2000 was cloudy resulting in sudden drops in incident radiation, when the sun was covered by clouds, but also in some peaks due to cloud reflectance. The overall doses during the experimental periods were similar on both days (Table 1).

The assemblages used in the experiments consisted of a variety of green algae, including *Gloeocystis* sp., *Chlorella* sp. and *Botryococcus* sp., Cyanobacteria, in-

Table 1. Accumulated UV-B dose at the surface during the experiments[a].

Date	Exposure time		
	4 h	8 h	20 h
22 February 2000	14.6 kJ	25.2 kJ	25.7 kJ
25 February 2000	13.6 kJ	23.6 kJ	24.0 kJ

[a] The accumulated dose at 85 cm depth was not determined, so that exact figures cannot be given. However, due to the high absorption of the basin water in the UV-B range (Fig. 1) it was surely < 2.5 kJ at 20 h exposure time.

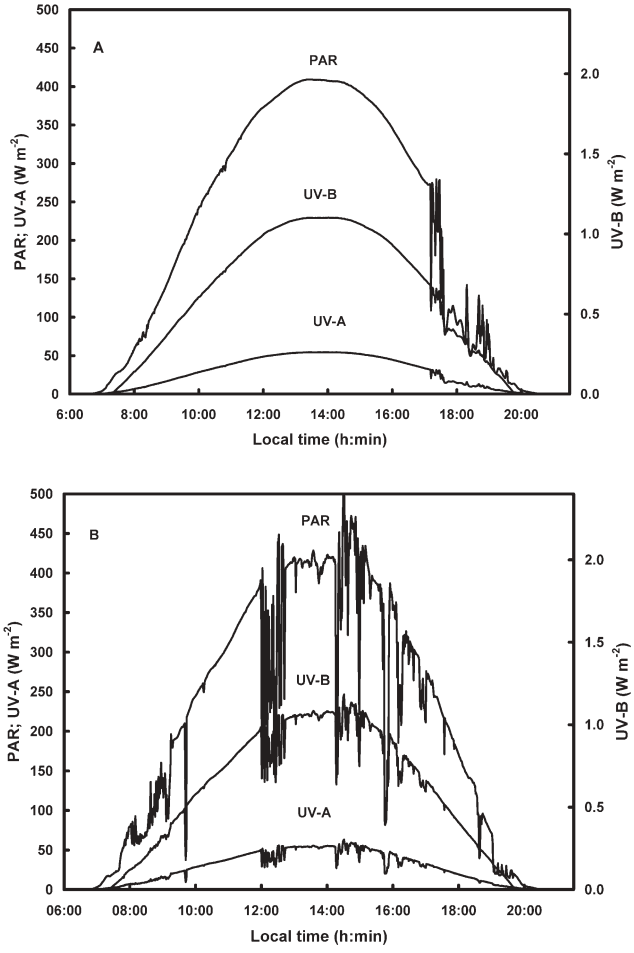

Figure 2. Irradiance in the PAR, UV-A and UV-B wavebands at the experimental site on 22 (A) and 25 February 2000 (B).

cluding *Croococcus* sp. and *Anabaenopsis* sp., and diatoms including *Amphora* sp. and *Navicula* sp. as determined by microscopic analysis. The chlorophyll *a* + *b* concentration was 360 µg L^{-1}.

Immuno-dot-blot analysis of T^T using an alkaline phosphatase coupled secondary antibody and BCIP/NBT as a substrate led to an uneven and strong background signal (data not shown). The background signal was restricted to the sample spots on the membrane and showed a slightly brownish discoloration as compared to the typical purple color produced by the reaction of BCIP/NBT with alkaline phosphatase. We repeatedly observed such a phenomenon with this detection system. We interpret this behavior as a side reaction of the substrate mixture with some remaining enzymatically active polypeptides on the membrane.

After stripping of the antibodies and reincubating the membrane, dot-blot analysis of the samples revealed an induction of thymine dimers by some of the treatments. From the image of the blot for both experiments (Fig. 3), it is clearly seen that the luminescence is restricted to the DNA spots that have been exposed to full solar radiation at the surface level. During the first experiment (February 22nd), there was a significant ($p < 0.05$) damage to the DNA of the samples exposed at the surface as a function of the exposure. Initial values of T^T were relatively low (3.2 ± 0.9 T^T Mbp^{-1}), but after the first 4 h of exposure thymine dimers were already formed (Fig. 4) reaching a value of 25.5 ± 2.6 T^T Mbp^{-1}. During the subsequent 4 h, the DNA was damaged even further, and the concentration of thymine dimers increased to 50.9 ±

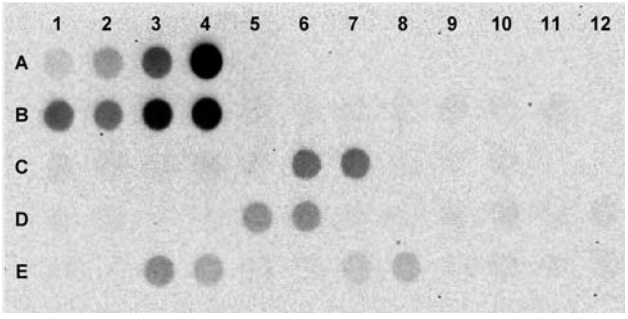

Figure 3. Dot blot of DNA extracted from a natural phytoplankton assemblage exposed to solar radiation for different durations. The gray values of the image were inverted for better visibility (equivalent to a negative image). Row A: Standard curve prepared from artificially irradiated plasmid DNA (pBSK). A 1–4: plasmid DNA containing 2.2×10^{11}, 5.5×10^{11}, 1.1×10^{12} and 3.3×10^{12} thymine dimers, respectively. Rows B and C: Experiment conducted from 22–23 February 2000. Rows D and E: Experiment conducted from 25–26 February 2000. Samples before irradiation: C 1–3 and D 1–2 and D 11. Samples exposed at surface level for 4 h: B 1–2 and D 5–6. Samples exposed at surface level for 8 h: B 3–4 and E 3–4. Samples exposed at 85 cm depth for 4 h: B 5–6 and D 7–8. Samples exposed at 85 cm depth for 8 h: B 7–8 and E 5–6. Samples exposed to PAR and UV-A only for 4 h: B 9–10 and D 9–10. Samples exposed at surface level after recovery overnight: C 6–7 and E 7–8. Samples exposed at 85 cm depth after recovery overnight: C 8–9 and E 9–10. Samples exposed to PAR and UV-A only after recovery overnight: B 11, C 10 and E 11–12. Samples kept in darkness after 4 h: D 3–4. Samples kept in darkness after 8 h: E 1–2. Samples kept in darkness up to the end of the experiment: C 4–5.

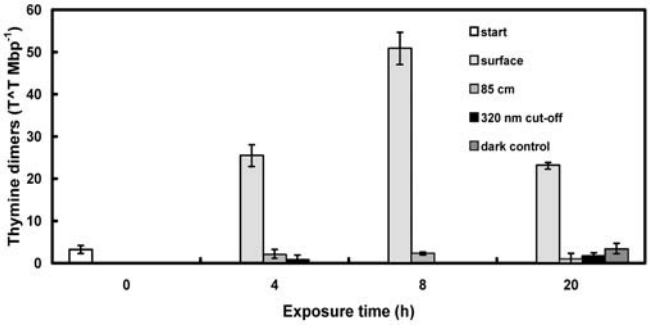

Figure 4. Frequency of thymine dimers in a natural freshwater phytoplankton assemblage exposed to natural solar radiation for different durations at the surface of a water basin, or at 85 cm depth. The exposure time of 20 h included a dark period of 10 h. Means of 2–3 replicates ± 1 standard deviation.

3.8 T^T Mbp^{-1}. There was no significant ($p > 0.05$) induction of T^T in all the samples incubated at 85 cm depth as well as in those incubated at the surface covered by a 320-nm filter. Similar results were obtained in the experiment conducted on February 25th, with the exception that T^T levels did not significantly change (data not shown) during the afternoon hours. In both experiments there was a repair of T^T dimers during the dark period although this process was not complete, and by the next light period the amount of dimers was 23.2 ± 0.6 T^T Mbp^{-1} in the first experiment.

Discussion

The data obtained in the present study support the hypothesis presented in the Introduction and show that natural solar radiation can damage phytoplankton DNA causing the formation of thymine dimers (Buma et al., 2001a; Buma et al., 2001b; Helbling et al., 2001). Additionally we found that stripping off the primary and secondary antibodies and reprobing the blot, using a chemoluminescence detection system, allowed us to avoid strong background signals that occurred with the colorimetric detection based on BCIP/NBT.

The formation of thymine dimers in DNA is dependent on the irradiance/dose received by the cells (Buma et al., 1995). In addition to the variations of solar radiation during the day (i.e., changes in solar zenith angle, clouds, aerosols, etc.), the depth at which the cells are incubated plays an important role in determining accumulation of thymine dimers, as shown for marine phytoplankton from Antarctica (Buma et al., 2001a) as well as from mid latitudes (Buma et al., 2001b). Similar results were found in this study using natural freshwater phytoplankton assemblages that were exposed in a water basin enriched with humic substances, in an attempt to mimic the situation in a natural freshwater body and simultaneously to quantify the doses of solar radiation by using the data from a broadband filter radiometer. Exposure to surface level solar radiation led to considerable accumulation of thymine dimers, under near *in situ* conditions. After prolonged exposure at the surface and accumulation of considerable amounts of thymine dimers (50.9 T^T Mbp^{-1}), some but not total recovery occurred overnight (23.2 ± 0.6 T^T Mbp^{-1}). Thymine dimers can be photorepaired by the enzyme photolyase using the energy of UV-A or visible light at temperatures high enough to permit the action of this enzyme (Sancar, 1996). This likely happened during evening hours, when UV-B was weaker, but some PAR and UV-A were still available (UV-B became undetectable at 7:43 pm, whereas UV-A and PAR were measurable till 8:40 pm).

Another mechanism to repair DNA is the nucleotide excision repair that does not require the presence of light (Britt, 1996). This mechanism might have come into effect during the night, further reducing the frequency of thymine dimers. The observed repair of DNA damage over night is in contrast to observations in some other studies. Helbling et al. (2001) found the formation of similar amounts of CPDs in natural freshwater phytoplankton assemblages upon exposure to solar radiation at the surface of Lake Titicaca, but little or no recovery was observed overnight. This might be due to the higher total dose received during surface incubations in this high altitude tropical lake. In a different study (Buma et al., 2001b), undertaken at a site relatively near to our study area, high initial levels of CPDs were found in post-bloom marine picoplankton assemblages taken in the morning, and no repair was observed in samples that were incubated at the surface, but some repair was evident in deep samples at the end of the incubation period. However, results similar to ours were found in marine picoplankton from the Red Sea (Boelen et al., 2002).

The phytoplankton community investigated in this study seems to be relatively UV-tolerant with respect to DNA damage, at least when compared with its marine counterpart at the same latitude. However, removal of thymine dimers was incomplete in the samples exposed at the surface suggesting that the repair capacity, of at least some of the organisms in the population, might be insufficient to allow survival at the surface. There are, however, at least two factors that need to be taken into account when extrapolating the present results to the ecological situation in the lake. First, the low initial level of DNA damage indicates that in their natural situation in the lake the algae were well protected from UV-B even at a depth of only about 10 cm. This might be attributed to the attenuation of solar radiation by high DOM concentrations typical for eutrophic lakes (Morris et al., 1995) and the high phytoplankton density in this lake as indicated by the high chlorophyll concentration (360 μg L^{-1}). Second, in the experimental situation, the organisms were limited in their ability to migrate or be passively circulated within the water column (i.e., they were incubated at a fixed depth), while under natural conditions the situation might be less critical due to vertical mixing and active migration. Samples that were incubated at 85 cm depth showed no induction of DNA damage, indicating that humic substances in the water provided an effective UV protection to the phytoplankton organisms, thus supporting the fact that UV-B levels in the lake might be insufficient to substantially damage the DNA, and cells would only be damaged when carried to the surface by vertical mixing.

This study clearly revealed that solar UV-B radiation induced the formation of thymine dimers in a freshwater phytoplankton assemblage exposed at the water surface. Elevated levels of UV-B radiation might lead to an enhanced formation of DNA lesions in fresh water phytoplankton due to the higher effectiveness of short wave-

lengths in the formation of thymine dimers (Buma et al., 1997). However, in their natural habitat the organisms might not experience such severe damage, as high concentrations of DOM and algae, together with vertical mixing processes might prevent the cells from receiving excessive levels of UV.

Acknowledgments

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