Accumulation of damaged bacteria and viruses in lake water exposed to solar radiation

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ABSTRACT: Ambient ultraviolet radiation (UVR) is known to have direct negative effects on aquatic bacteria at the molecular level, damaging DNA, and at the level of community metabolism, but little is known about the effects of UVR at the cellular level. In order to study the direct and indirect effects of UVR on bacterioplankton, we developed a staining protocol based on the exclusion nucleic acid stain TOPRO-1 that allows the detection of cells with damaged membranes. We used this protocol to assess the effect of UVR in water samples collected from Lac Cromwell, a small dystrophic Canadian Shield lake. Samples were incubated 5 d *in situ* in a series of dialysis bag experiments under natural solar radiation. Samples with and without grazers were suspended in the lake's epilimnion and received graded amounts of ambient UVR. Abundances of bacteria, viruses and heterotrophic nanoflagellate grazers, and the number of TOPRO+ cells were monitored. In samples exposed to full solar radiation, a marked increase in the number of bacteria that took up the exclusion nucleic acid stain TOPRO, an indicator of cellular damage, was observed. The rate of accumulation of damaged bacteria reached a maximum of 13.5 % d⁻¹ and was a direct function of the UVR intensity in solar radiation. There was also an increase in the mean total viral abundance in bags exposed at the surface. We hypothesize that this increase in viral number was due to the induction by UVR of the lytic cycle in lysogenic bacteria. In the presence of grazers, however, the virus-to-bacteria ratio was lower and the bacterial mortality attributable to viral lysis was reduced by half, from 10.7 to 4.6 % d⁻¹. These observations strongly suggest that grazers and viruses are competing for the same bacteria and that grazers may play an important role in regulating the proportion of bacterial mortality caused by viruses. Our results show that high intensities of ambient UVR, particularly in the surface waters of lakes with shallow epilimnia or those that undergo periods of microstratification, may be an important factor influencing bacterial mortality, either directly or indirectly via the induction of lysogenic bacteria.

KEY WORDS: Lake · Viruses · Bacterioplankton · Ultraviolet radiation · Lysogeny · TOPRO · Membrane damage · Bacterial mortality · *In situ* incubation · Dialysis bags

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INTRODUCTION

The effect of ultraviolet radiation (UVR) on aquatic microorganisms has been the focus of recent research interest, following demonstrable increases in the level of UVR reaching the earth's surface associated with the depletion of the stratospheric ozone (Madronich et

al. 1995). UVR may have diverse negative effects on aquatic biota, including species composition changes in algal communities, which in turn may affect trophic interactions that alter entire food webs (Vincent & Roy 1993, Bothwell et al. 1994, Williamson 1995, Mostajir et al. 1999). More research emphasis has been placed on autotrophic organisms because of their fundamental importance in food webs and their dependence on light for photosynthesis; relatively less work has been done on the effects of UVR on natural communities of aquatic bacteria. Yet, because of their significant role

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in carbon and nutrient fluxes, the effects of UVR on bacterial communities and their predators are also of considerable interest.

The effects of UVR exposure on microbial communities are very diverse and include both negative and positive influences over metabolism and growth. Bailey et al. (1983) observed a light-associated decrease in bacterial activity as measured by amino acid uptake rates in unfiltered seawater samples. This inhibition was associated with both UV and visible light. Sieracki & Sieburth (1986) showed that sunlight did not have a significant effect on the growth rate of bacterioplankton, but it extended the lag phase of the incubated communities, suggesting that the bacteria were more susceptible to sunlight during protein synthesis prior to exponential growth. Herndl et al. (1993) reported large decreases in the rate of leucine incorporation by bacteria. These authors suggested that the rate of protein synthesis is more rapidly inhibited by exposure to UVR than is DNA replication. Similarly, Wilhelm & Smith (2000) observed an important daytime photo-inhibition of bacterial production as measured using thymidine and leucine incorporation in the upper 5 m of Lake Erie. Some of these effects may result from direct UV-induced cellular damage, but others may result from indirect effects. For example, it has also been demonstrated that the addition of low concentrations of hydrogen peroxide, a toxic photoproduct of UVR, can have a strong negative effect on the protein production rate of a freshwater bacterial community (Xenopoulos & Bird 1997).

Sunlight can also have positive effects on the growth of aquatic bacterial communities, through the photochemical degradation of refractory dissolved organic carbon (DOC) into more bioavailable forms (Kieber et al. 1989, Moran & Zepp 1997). This photochemical breakdown stimulates bacterial growth in highly colored lakes (Lindell et al. 1995, Wetzel et al. 1995, Bertilsson & Tranvik 1998). But recent evidence suggests that, at least in some cases, the photochemical transformation of DOC can reduce its bioavailability to bacterioplankton (Tranvik & Kokalj 1998, Obernosterer et al. 1999, 2001). UVR may explain diurnal variations in fractions of the DOC in marine environments (Burney et al. 1982, Müller-Niklas et al. 1995), but it has been suggested that the buildup of organic matter in the afternoon was due to sunlight-induced inhibition of the bacterial community (Burney 1986).

Not only bacteria are affected by UVR, but other components of the microbial food web are as well. The rate of flagellate grazing on bacteria may also be reduced when the organisms are exposed to UVR (Sommaruga et al. 1996), suggesting that UVR may indirectly affect loss rates of bacteria in surface waters. Solar radiation may also influence the interactions

between viruses and bacteria in aquatic systems in diverse ways. UVR generally has a negative effect on aquatic viral abundance and infectivity (Suttle & Chen 1992, Noble & Fuhrman 1997, Wilhelm et al. 1998a,b), but it has also been identified as an important inducing agent of the lytic cycle in lysogenic bacteria (Freifelder 1987), with an increase in both bacterial mortality and viral production. Evidence exists for the presence of both lytic (Wilcox & Fuhrman 1994, Weinbauer & Suttle 1996) and lysogenic (Ogunseitan et al. 1992, Jiang & Paul 1994, 1996, Tapper & Hicks 1998, Cochran et al. 1998, Weinbauer & Suttle 1999) phage in natural aquatic communities. The relative importance of these viral reproductive strategies among systems remains uncertain (Wilson & Mann 1997). Unnatural inducing agents have commonly been used in these studies (Wilcox & Fuhrman 1994, Weinbauer & Suttle 1996, Tapper & Hicks 1998) since the primary objective has not been to determine their natural rate of induced lysis but to determine the proportion of lysogens in the system.

It is likely that the positive and negative effects of UVR occur in parallel and through very different pathways. For example, it is likely that UV exposure directly results in cell damage or death, although not all bacterioplankton seem to be equally susceptible to UV damage (Joux et al. 1999, Arrieta et al. 2000). UVR may also result in cell death through the production of toxic substances such as hydrogen peroxide or by triggering the lytic cycle in lysogenic bacteria. But it is also possible that the same level of UV exposure ultimately leads to an increase in overall community protein synthesis by increasing the bioavailability of more organic substrate. Thus, the effects at the single-cell level may not necessarily correspond to the longer-term effects at the community level. Indeed, most studies to date have assessed the effects of UVR on aquatic bacteria at the level of bulk metabolism, but the underlying mechanisms have not been fully explored.

The first objective of the present study was to assess the possible damaging effects of exposure to solar radiation on lake bacterioplankton assemblages at the single-cell level. For this purpose, we developed a staining protocol that allows the detection of damaged cells with compromised cellular membranes, based on the exclusion nucleic acid stain TOPRO-1. Because cell damage may result from direct UV effects, or from the UV-induced enhancement of bacterial mortality due to viral lysis, we have also investigated the dynamics of viral populations in our experimental systems. In particular, our second objective was to explore whether the effects of exposure to solar radiation on bacterial integrity were accompanied by variations in viral abundance and in the virus-to-bacteria ratio (VBR) that could be interpreted as induction of the lytic cycle in

lysogenic cells. Finally, we also wanted to determine whether the presence or absence of bacterial grazers influenced the patterns of UVR effects on bacteria and viruses. We addressed these questions in 2 series of dialysis bag experiments conducted *in situ* in a colored lake, where natural bacterioplankton assemblages were exposed to different levels of UVR in natural sunlight.

MATERIALS AND METHODS

Site and sampling dates. The experiments were carried out in Lac Cromwell, a small, dystrophic lake located on the Canadian Shield, NE of Montréal, Québec, Canada (9 m maximum depth, 8 ha, 7 to 13 mg DOC l⁻¹, 46°N, 74°W). Temperature profiles were taken every day during the experimental period and indicated that the lake was stratified at a depth of 2 m. The temperature of the epilimnion varied between 24.5 and 25°C in July and between 19.1 and 20°C in August. Ambient UVB radiation was measured using a Brewer spectrophotometer (instrument number MKIV 79) at 45°47'N, 73°75'W. These data were weighted according to the McKinlay-Diffey erythral action spectrum and were kindly provided by Environment Canada. The erythral action spectrum was used due to its availability in the World Ozone and Ultraviolet radiation Data Centre (WOUDC) database. However, use of the erythral over the more germane Setflow DNA action spectrum may have de-emphasized the differences in UV flux between dates.

Experimental design. Experiments were carried out *in situ* using dialysis bags (Spectra/Por 4, 7.5 mm flat width, molecular weight cut off 12 000 to 14 000 Da). The bags allow diffusion of nutrients and organic matter, thus circumventing the effects of nutrient exhaustion in longer-term incubations. Bags were cut to a length of 24 cm, thoroughly washed in hot tap water, rinsed overnight, and then soaked for at least 3 h in distilled water, before the start of the experiments. The washed bags were then clamped on one end. Water used for the experiments was collected at 1 m depth in the lake and prefiltered at 200 µm to remove large zooplankton.

In the first experiment (July 25 to 30, 1995), 30 dialysis bags were filled with approximately 300 ml of this water to make the '200 µm' or 'grazer' treatment. Another part of the lake water was further filtered, first through glass fiber filters (Gelman AE, 1.2 µm), then through Nuclepore polycarbonate membranes (0.8 µm) to eliminate heterotrophic nanoflagellates (HNFs) and other bacterial grazers. An equal number of bags (30) were filled from this water fraction to make the '0.8 µm' or 'non-grazer' treatment. Upon verifica-

tion, we found no growth of nanoflagellates in our 0.8 µm filtered water during the incubation period. Although the 200 µm filter may have removed some of the larger ciliates, the nanoflagellate community remained relatively intact as compared with lake water samples. Triplicate bags of each of the grazer (200 µm) and non-grazer (0.8 µm) treatments were suspended in a plastic cage, through which the epilimnetic water could freely circulate, directly below the surface of the water (UVR + photosynthetically available radiation [PAR] treatment) and in another cage moored at 1.5 m depth (Deep treatment), above the thermocline. Overall, 5 cages were set up at the surface and 5 at depth. Triplicate bags from the 2 depths (UVR + PAR and Deep) and the 2 filtration treatments (with and without grazers) were retrieved daily for 5 consecutive days between 10:00 and 11:00 h.

Spectrophotometric measurements showed that the dialysis bags admitted at least 80 % of UVA and 70 % UVB (but probably more once corrected for scattering in the spectrophotometer); the bacteria in the subsurface bags therefore received UVR of slightly attenuated intensity. The 1 % UVA and UVB depths were estimated at 0.56 and 0.21 m, respectively, using a Lac Cromwell-specific model developed by Scully & Lean (1994). Thus, bags moored at 1.5 m depth received approximately 10 % of surface light but no significant amount of UVR.

For the second experiment (August 25 to 30, 1995), an additional light treatment was used. A third set of 5 cages were covered with a sheet of 0.6 cm wide Plexiglas, which eliminated 100 % of all UVB (280 to 320 nm), 100 % of UVA up to 365 nm, and approximately 60 % of UVA 365 to 400 nm, but allowed PAR to pass. These cages were also suspended from the surface, and we refer to these bags as the PAR treatment. Thus, in the second experiment there were 3 light treatments (UVR + PAR, PAR and Deep), and for each of these, there was a grazer and non-grazer treatment. As in the first experiment, triplicate bags for each of these 6 treatments were sampled daily for 5 d, in addition to the samples from the surrounding ambient waters.

Hydrogen peroxide concentrations were measured daily in all treatments and in the ambient waters using the technique described by Miller & Kester (1988). Briefly, a 30 ml sample was collected and the hydrogen peroxide was fixed in the field using the following reagents: peroxidase (235 purpurgallon units l⁻¹, final), 5 µM (*p*-hydroxyphenyl) acetic acid (POHPAA) final and 4.9 mM Tris buffer final. Samples were kept in the dark and at 4°C until processing. Immediately upon return to the laboratory, samples were brought to room temperature and read spectrophotometrically (λ_{ex} = 313 nm, λ_{em} = 400 nm). Spectrophotometric readings

were converted to *in situ* concentrations relative to a standard curve with known concentrations of hydrogen peroxide, ranging from 0 to 1 nM, and using the same fixation procedure as *in situ* sampling. Theoretically, since all sample bags were suspended in the lake's epilimnion, all treatments were subjected to the same concentration of hydrogen peroxide (Scully & Vincent 1997) given the rapid rate of diffusion across the dialysis bags. Therefore, since all bags were subjected to the same amount of oxidative stress, differences between the treatments were a result of direct UVR effects.

Enumeration of total bacterial and viral abundance.

The total number of bacteria in the bags and ambient water was determined within 2 h of collection on unfixed samples. Samples were stained with SYTO-13 and total abundance was determined by flow cytometry using a FACScan (Becton Dickinson) equipped with a 15 mW, 488 nm air-cooled argon-ion laser (del Giorgio et al. 1996a).

Samples for virus counts were fixed using 1% glutaraldehyde, prepared on the day of collection, and enumerated at a later date. Viruses were pelleted directly onto 400-mesh formvar-coated copper grids at $100\,000 \times g$ for 30 min using an EM-90 rotor in a Beckman airfuge (Hammond et al. 1981). Grids were air-dried, stained with 2% uranyl acetate for 5 min and counted directly using a Philips transmission electron microscope (EM) at a magnification of 90 000 $\times$. Recovery efficiency using this method has been established at 96% (Maranger et al. 1994). Viral abundance was estimated by observing 50 fields per EM grid. The number of viruses observed per grid was approximately 60 to 180. The coefficient of variation (CV) of the mean count per sample was less than 10%.

Enumeration of 'damaged' bacterial cells. We developed a staining protocol that allows the enumeration of bacteria with compromised cell membranes in natural lake water samples. The protocol is based on the exclusion by healthy cells of the nucleic acid stain TOPRO-1, which penetrates cells with compromised membranes, strongly binding to nucleic acids. TOPRO-1 is a monomeric cyanine nucleic acid stain that has an absorption maximum of 515 nm and an emission maximum of 531 nm. Fluorescence is greatly enhanced upon RNA and DNA binding, and the fluorescence of unbound dye is negligible. The relatively high molecular weight of TOPRO-1 (645 Da) and the structure of the cationic cyanine monomer are such that the dye can only enter cells with damaged or 'leaky' membranes and cannot enter cells with intact membranes (Haugland 2000). TOPRO-1 is readily excited with the argon laser line at 488 nm, and emits in the green. Stained cells that are presumably injured or dead can be easily counted using either epifluores-

cence or flow cytometry; the latter was used in these studies.

In preliminary experiments we optimized the staining protocol and tested its effectiveness to confirm that this fluorescent marker is responsive to treatments inducing various degrees of cellular stress. The final protocol that was used for all experimental samples was the following: 1 ml of fresh sample was dispensed into a flow cytometry tube and TOPRO-1 (Molecular Probes) was added to a final concentration of 1 μ M. The samples were vortexed and incubated for 2 min, after which the samples were amended with green fluorescent microbeads (1.0 μ m) and run in the flow cytometer at the lowest flow rate (approximately 12 μ l min⁻¹). TOPRO-stained cells were detected in a cytogram of side scatter versus green fluorescence, with the same parameters that were used to enumerate SYTO-stained cells. Approximately 4000 to 10 000 events were acquired, but the total processing time (incubation + cytometric analysis) was always kept below 5 min. Our preliminary experiments showed that there was some non-specific staining of TOPRO on cell surfaces, and this could become significant in terms of overall numbers after 10 to 15 min of total processing time. Short incubation times (<10 min) resulted in highly repeatable measurements and, provided that processing time was within these limits, the average CV of TOPRO+ cells counts was roughly similar to that of total counts using SYTO-13 (del Giorgio et al. 1996a).

Several experiments were carried out, using the above protocol, to assess the performance of TOPRO-1 as a marker of cells with damaged membranes, both in cultures and in natural bacterioplankton assemblages. Cells were exposed to factors that induce cell stress and injury, including exposure to the ionophore gramicidin S, heat and ultrasound, and the change in the proportion of cells stained with TOPRO-1 after each treatment was determined using flow cytometrically. Membrane permeability of cells was altered by exposure to gramicidin S, which depolarizes cells by making channels through the membranes (Lopez-Amorós et al. 1995). Cultures of *Escherichia coli* (WL 66) were grown in tryptic soy broth (Sigma Scientific). Cells were sampled during exponential growth and diluted to a final concentration of 10^6 cells ml⁻¹. Aliquots of this dilute culture were exposed for 10 min to varying concentrations, ranging from 0 to 60 μ g ml⁻¹, of gramicidin S (Sigma Scientific). The total number and the number of TOPRO+ cells were determined cytometrically. Fresh lake water samples (lakes Brome and Cromwell, Québec) were either incubated at 60°C or sonicated at 50 W (Braun Sonicator equipped with a microtip), and the total number of bacteria and the number of TOPRO+ cells were determined at different incubation

or sonication times. The staining protocol using TOPRO-1 and SYTO-13 was also compared with a commercial product, BacLight®, a staining kit developed by Molecular Probes to enumerate dead and live bacteria. This kit consists of a proprietary mixture of nucleic acid stains that selectively stain live or dead cells and has been tested in a variety of cultured Gram-positive and -negative bacteria (Haugland 2000). Fresh and heat-killed (60°C for 15 min) lake samples were stained with TOPRO-1 and then SYTO-13, or with the BacLight kit, and total and dead cells observed with each method were counted with epifluorescence. Triplicate preparations were made for all samples.

Rate estimations: TOPRO+ accumulation and bacterial damage. An exponential increase in the number of damaged bacteria during the experiment was observed. Hence, the accumulation rate of TOPRO + bacteria ($T+$, d^{-1}) was calculated from the difference in TOPRO bacterial abundance between Day 0 and either Day 3 or 4 using the following exponential equation:

$$T+ = (\ln T_t - \ln T_0)/t$$

where T_t and T_0 are the number of TOPRO cells (ml^{-1}) after the incubation period and at time zero, respectively, and t is the incubation time in days. The incubation time was equal to the time taken to reach the maximum abundance of TOPRO cells. This rate of accumulation of TOPRO+ cells, however, was independent of the total amount of bacteria during the incubation period and did not provide us with an estimate of the proportion of cells that were damaged per day. In order to estimate the proportion of the community that was damaged per day relative to the total community, we calculated a damage rate (DR, % d^{-1}) for each day using the following equation:

$$DR = (T_{t+1} - T_t)/B \times 100\%$$

where the difference $T_{t+1} - T_t$ is the number of additional TOPRO+ cells after 1 d of incubation and B is the average bacterial abundance between those 2 d. The final DR estimate was taken as the mean of daily estimates up until Day 4 or when the number of TOPRO+ cells stabilized.

Statistical analysis. All statistical analyses were done using SAS (SAS Institute). The 5 d means of the sample treatments were compared using 1-way and 2-way ANOVA. A Tukey test was performed to identify the significant differences between the treatments for all the 1-way analyses and non-interactive terms in the 2-way ANOVA. The daily means of the triplicate bags of each treatment were used in the correlation analysis. No transformations were necessary to meet the normality assumptions of correlation and ANOVA, with the exception of the VBR, where data were log transformed to meet normality assumptions of the variances.

RESULTS

Effectiveness of the TOPRO protocol

Initial tests were carried out to determine the optimum concentration of TOPRO-1 and time of incubation that would minimize the effect of non-specific binding. It was determined that a range of dye concentrations between 0.1 and 5 μM yielded similar and repeatable results, as long as the total processing time was kept below 10 min. As has been shown before for SYTO-stained cells (del Giorgio et al. 1996a), there was good agreement between cytometric and epifluorescence counts of 20 samples stained with TOPRO-1 (no significant differences, $p > 0.05$, data not shown), suggesting that cytometric counts are not significantly biased relative to epifluorescence counts. The average CV of triplicate cytometric counts of TOPRO-stained cells was 2.1%, which is similar to that obtained for total counts using SYTO-13 staining, and substantially lower than that for the equivalent triplicate epifluorescence counts (CV = 14%).

We assessed the effect of gramicidin, sonication and heating on the proportion of TOPRO+ cells in cultures as well as in bacterioplankton samples. A pure culture of *Escherichia coli* in exponential growth was extremely sensitive to exposure to gramicidin. This culture showed an almost negligible number of TOPRO+ cells before addition of gramicidin (<1% of the total count). Exposure of the *E. coli* culture to increasing concentrations of gramicidin resulted in sharp increases in the proportion of TOPRO+ cells (Fig. 1A). In the more drastic treatment with gramicidin (60 $\mu g\ ml^{-1}$), over 70% of the total cell count took up TOPRO (Fig. 1A), suggesting that intact *E. coli* cells were indeed impermeant to the stain, whereas gramicidin effectively resulted in the permeability of the cell membranes. Relatively gentle sonication of natural lake bacterioplankton samples had a marked effect on the proportion of cells that took up the TOPRO stain. There was a sharp increase in the proportion of TOPRO+ cells with time of sonication at 50 W (Fig. 1B), from 12% in the fresh lake sample, to over 70% after 40 s of continuous sonication. The total number of cells in the samples also increased (by up to 14%) with sonication time, possibly the result of both disaggregation of bacterial clumps and breakage of cells. Mild heating also had a marked effect on the proportion of TOPRO+ cells in bacterioplankton samples (Fig. 2A). There was a steady increase in the proportion of TOPRO+ cells, from 12 to over 50%, when a freshwater bacterioplankton sample was incubated at 60°C for up to 20 min (Fig. 2A). As with the sonication treatment, there was also a slight (up to 10%) increase in the total bacterial count following the heat treatment. The pro-

Fig. 1. Effect of (A) exposure to gramicidin S on the proportion of TOPRO+ cells in a culture of *Escherichia coli* and (B) sonication time at 50 W on the proportion of TOPRO+ cells in natural bacterioplankton. Error bars represent the average standard deviation ($\pm$ SD) of triplicate cytometric counts

Fig. 2. (A) Time course of the proportion of dead cells stained with TOPRO-1 and the proportion of active cells in samples heated at 60°C. Active cells were measured as those capable of reducing the tetrazolium salt CTC to its fluorescent formazan. (B) Changes in the rates of ^3H -leucine uptake with incubation time at 60°C of the above samples. Error bars represent ± 1 SD of triplicate measurements of each of the 3 variables

portion of respiring bacteria (Fig. 2A) and the rates of leucine uptake (Fig. 2B) concomitantly declined in the same sample with incubation time, so there was an inverse relationship between the proportion of TOPRO+ cells and both the proportion of active bacteria (cells that reduce 5-cyano-2,3 ditolyl tetrazolium chloride, CTC+) and their uptake rates. Although the samples incubated for 20 min at 60°C had no measurable rates of leucine uptake, there were still some cells that reduced CTC ($<1\%$) in the sample, and the proportion of TOPRO+ cells remained below 60%.

We compared our protocol using TOPRO-1 and SYTO-13 with a commercial product, BacLight® (Molecular Probes), specifically designed to enumerate live and dead bacteria (Boulos et al. 1999). Lake water samples were heated at 60°C for 20 min, and the fresh and heat-killed samples were stained with either TOPRO-1 and SYTO-13 following the protocol described above, or with the reagents in the BacLight® kit, following the manufacturer's instructions. Triplicate preparations of each method were counted under epifluorescence. There were no significant differences between the number of live and dead cells estimated using the 2 methods in the fresh samples (average proportion of dead cells was $19 \pm 6\%$; Fig. 3). Following the heat treatment, the proportion of dead cells greatly increased as estimated by either method, although our protocol yielded somewhat higher proportions of dead cells ($75 \pm 3\%$) than the commercial product ($65 \pm 4\%$) (Fig. 3). Overall, however, there was good agreement ($<10\%$ difference) between the 2 staining protocols.

These results collectively suggest that TOPRO-1 staining is generally not taken up by healthy bacterial cells but quickly stains cells that have undergone even mild levels of stress. Cells stained with TOPRO-1 may be easily enumerated with both epifluorescence and flow cytometry, and short incubations and low dye

Fig. 3. Proportion of dead cells in fresh and heat-treated (60°C for 20 min) bacterioplankton samples determined using the TOPRO-1 protocol or a commercial product (BacLight) developed by Molecular Probes to discriminate between live and dead cells. Error bars represent ± 1 SD of triplicate epifluorescence counts of each preparation

concentrations minimize the non-specific binding of TOPRO-1 to cellular surfaces. In this paper we will refer to the TOPRO-stained bacteria simply as either TOPRO+ or 'damaged', because we can not conclusively state that TOPRO+ cells are all dead (i.e. repair may be possible). But we should emphasize that all of the samples that were used to establish TOPRO counts were handled in an identical manner, and therefore we propose that the relative differences in TOPRO+ cell abundance presented in this study provide a useful cell-specific index of cell integrity and damage.

Experimental conditions

A major difference between the 2 experiments was that the mean ambient UVB levels during the July experiment were significantly higher than those observed in August (Table 1). This increase in UVB was not due to a decrease in stratospheric ozone (Table 1) and, as we discuss below, appeared to have consequences on bacterial and viral dynamics in the bags. Initial bacterial and viral abundance were similar for the most part between the filtered 200 μm and 0.8 μm samples (Table 1), with the notable exception of bacterial abundance in the second experiment. This 65% decrease in total bacterial abundance, due to greater removal by the 0.8 μm filtration step in the second experiment, resulted in an unplanned significant increase in the VBR in the grazer-free treatments in this experiment.

In order to verify the uniformity of mixing in the epilimnion and of diffusion between the bags and the surrounding waters we measured hydrogen peroxide in the bags and *in situ*. This photoproduct would vary between depths in a poorly mixed epilimnion or would be concentrated in the bags if the exchange with the local environment was not rapid enough. The concen-

Fig. 4. Daily changes in (A) TOPRO-stained bacterial and (B) viral abundance from the grazer-free treatments in August experiment. Standard errors ($\pm$ SE) reported. PAR: photosynthetically available radiation

tration of peroxide in samples taken between 10:00 and 11:00 h ranged from 80 to 150 nM and no significant difference in peroxide concentration was found between treatments. We conclude that indirect photochemical effects were eliminated by the experimental design and that any treatment effects were due to direct solar radiation damage.

The response of the microbial assemblages to the solar radiation treatments was quite rapid. The abundance of TOPRO+ bacteria increased exponentially in the treatments exposed to full sunlight and peaked for the most part on either Day 3 or 4 (Fig. 4A). Noticeable increases in viral abundance were sometimes observed within 24 h and generally peaked on Day 2 or Day 3 (Fig. 4B). Once values had peaked, abundance either decreased slightly or remained stable. Because we were most interested in the net effect of treatment on abundances, the day-to-day dynamics will not be presented in detail here; only overall treatment means and rates of change in viral and bacterial abundance will be discussed. Treatments include samples suspended just beneath the surface of the water and

Table 1. Mean and standard deviation of UVB radiation, UVB:UVA ratio, and ozone levels for different experiments. Initial concentrations of bacteria and viruses are also reported for the different filtered fractions

Variable	July	August
Erythematous UVB (J m^{-2})	761 ± 145	447 ± 57
UVB:UVA (300 nm:325 nm)	0.0095 ± 0.0009	0.0056 ± 0.001
Ozone ^a (DU)	317 ± 17	298 ± 9
Total bacteria (0.8 μm)	$2.7 \times 10^6 \text{ ml}^{-1}$	$1.7^a \times 10^6 \text{ ml}^{-1}$
Total bacteria (200 μm)	$3.7 \times 10^6 \text{ ml}^{-1}$	$5.2^a \times 10^6 \text{ ml}^{-1}$
Viruses (0.8 μm)	$7.6 \times 10^7 \text{ ml}^{-1}$	$1.3 \times 10^8 \text{ ml}^{-1}$
Viruses (200 μm)	$8.4 \times 10^7 \text{ ml}^{-1}$	$1.6 \times 10^8 \text{ ml}^{-1}$

^aOzone was calculated as the mean value for the second half of each experimental month

Fig. 5. One-way ANOVA of the 5 d mean with SE of viral abundance between treatments for (A) July ($F = 3.35$, $df = 4, 18$, $p < 0.03$) and (B) August ($F = 3.35$, $df = 6, 28$, $p < 0.01$). Letters denote significant differences ($p < 0.05$) between treatments using a Tukey test

exposed to solar radiation (UVR + PAR), samples moored at 1.5 m receiving no UV and very little PAR (Deep) and samples receiving only PAR and some UV (PAR), the latter only in the second experiment. For each of these 'light' treatments there was a grazer treatment (200 μm) and a grazer-free treatment (0.8 μm). 'Lake' refers to the measurements taken in the actual epilimnetic waters during the experiments. The viral and bacterial abundances were also determined in the ambient lake waters throughout the experiments, but these data were not included in the between-treatment comparison of rates of damaged cell accumulation because the abundance of TOPRO+ cells remained relatively constant in the surface waters.

Treatment effects on abundance

Using a 1-way ANOVA on the 5 d means, we observed a significant positive effect of UVR on viral abundance in both months (Fig. 5A, B). The greatest viral abundance was observed in the 0.8 μm filtered

UVR + PAR treatment, which was grazer free. Despite this, neither grazer absence nor presence, nor the interaction of grazing and UV, had any significant effect on total viral abundance. Only UVR + PAR had a positive effect on the viral abundance. In those samples exposed to ambient light conditions but protected by Plexiglas (Fig. 5B, treatments PAR 0.8 and PAR 200), viral abundance was intermediate between the samples from the deep waters (Deep) and those exposed to full solar radiation at the surface (UVR + PAR).

The number of TOPRO+ bacteria also increased markedly when samples were exposed to solar radiation (Fig. 6A,B). To correct for the possibility that this increase in damaged bacteria was simply due to an increase in total bacteria, we compared the percentage damaged bacteria between treatments. A 1-way ANOVA confirmed a significant positive effect of UVR on the accumulation of damaged bacteria relative to total cells (Fig. 6C,D). Although the pattern of the increase in the proportion of TOPRO+ cells was similar between experiments, it was interesting to note that the average proportion of damaged cells was significantly higher in the July experiment relative to August. We hypothesize that this difference was the direct result of the higher UVR levels that occurred in the July experiment.

The increases in viral abundance and in the number of TOPRO+ bacteria in response to the different treatments were positively correlated in both experiments (Fig. 7A,B). This suggests that these variables consistently respond in a similar fashion to solar radiation in this lake. The intensity of the ambient UVR had an effect on the strength of this response. Viral abundance and TOPRO+ bacteria were more tightly correlated in the July experiment when the intensity of UVR was highest ($r_{\text{July}} = 0.82$ vs $r_{\text{August}} = 0.43$) as compared to August.

Although there was no apparent grazer effect on total viral abundance, there was a significant negative effect of the nanoflagellate grazers on the observed VBR (Fig. 8A,B). With grazers present there was a significant decrease in the VBR in both experiments (2-way ANOVA: $F_{\text{July}} = 5.9$, $p < 0.001$; $F_{\text{August}} = 3.0$, $p < 0.005$). There was no significant interaction between grazing and UV exposure on the VBR. Higher viral abundance as a result of UV exposure in the 0.8 μm grazer-free treatment led to an increase in the VBR in the first experiment. The initial concentration of bacteria being lower in all of the 0.8 μm treatments in the second experiment contributed to the higher VBR in the non-grazer treatments on Days 1 and 2. However, with these initial values removed, the trend of a lower VBR in the presence of grazers was still observed ($p < 0.02$).

Fig. 6. One-way ANOVA of the 5 d mean with SE of TOPRO+ bacterial abundance between treatments for (A) July ($F = 14.1$, $df = 4, 18$, $p < 0.0001$) and (B) August ($F = 15.05$, $df = 6, 28$, $p < 0.0001$) and for the %TOPRO+ cells for (C) July ($F = 16.9$, $df = 4, 18$, $p < 0.0001$) and (D) August ($F = 7.2$, $df = 6, 28$, $p < 0.0001$). Letters denote significant differences ($p < 0.05$) between treatments using a Tukey test

Damaged bacterial dynamics

The rate of accumulation of TOPRO+ bacteria was strongly related to the exposure of solar radiation (Table 2). The rates of increase of damaged bacteria were a function not only of exposure to solar radiation but also of ambient UVR intensity (Fig. 9). Given that the ambient UVB intensity was higher in the July experiment (Table 1), bags exposed at the surface were exposed to considerably more harmful UVR than in the month of August. Indeed we observed almost a 2.5-fold difference in the accumulation rate of damaged cells of those samples incubated at the surface in July as compared with August (Fig. 9). The presence of grazers had no effect on the rate of accumulation of TOPRO+ cells (Table 2).

DISCUSSION

Regulation of the abundance and diversity of aquatic bacteria is thought to result from some combination of resource (bottom-up) and predatory (top-down) controls including viral mortality (see reviews of Ducklow & Carlson 1992, Pace & Cole 1994). Our study shows that density-independent factors outside this frame-

Fig. 7. Correlation between daily mean TOPRO+ bacterial and viral abundance for (A) July ($r = 0.82$, $n = 24$) and (B) August ($r = 0.43$, $n = 41$)

Fig. 8. One-way ANOVA of the 5 d mean with SE of the virus-to-bacteria ratio between treatments for (A) July ($F = 3.79$, $df = 4, 18$, $p < 0.02$) and (B) August ($F = 5.6$, $df = 6, 28$, $p < 0.001$). Letters denote significant differences ($p < 0.05$) between treatments using a Tukey test. Data were log transformed for ANOVA although arithmetic values are graphically represented

work can be responsible for large losses within the bacterioplankton and play a major role in microbial community dynamics. When lake plankton samples were subjected to solar radiation, there was substantial mortality manifest either as direct cellular damage measured as loss of membrane integrity by the incorporation of TOPRO or indirectly, we hypothesize, through the agency of the induction of viral lysis of lysogenic bacteria.

In the July experiment an average of 46% of the bacteria in the UVR + PAR non-grazer (0.8 μm) treatment and 30% of the bacteria in the UVR + PAR grazer (200 μm) treatment took up the TOPRO dye as compared to the experiment carried out in August where only an average of 14 and 17% of the community in these respective treatments were TOPRO+ during the incubation. This increase in the proportion of damaged cells appears to be linked to the significantly higher amount of UVB measured in the July experiment. Not only was the intensity of the UVB higher, but the ratio of UVB:UVA was also significantly higher in the month of

Fig. 9. Rates of bacterial death (d^{-1}) measured using TOPRO given different light and UV exposure and intensities. Letters denote significant differences in rates between treatments using a Tukey test in a 1-way ANOVA ($F = 46.3$, $df = 4, 22$, $p < 0.0001$)

July (Table 1). Since both forms of light affect bacterial metabolism, it may be the ratio of both that determines the global impact on the bacterial community. UVA plays an important role in enabling photo-reactivation repair, whereby important enzymes such as photolyase are activated (Jagger 1973, Freifelder 1987). Photolyase actively cleaves the cyclobutane pyrimidine dimers (CPDs) formed by the harmful UVB exposure and restores the cell's activity. UVA has been demonstrated to play an important role in the recovery of native aquatic bacteria (Kaiser & Herndl 1997) and viruses (Weinbauer et al. 1997, Wilhelm et al. 1998a,b). Therefore, cells incubated in July were exposed to a greater proportion of harmful rays without the equivalent photoreactivation repair benefit. One of the main concerns about a decrease in ozone thickness is that the increase in harmful UVB will not be matched by an increase of the more beneficial

Table 2. One-way ANOVA results between treatments for July and August, showing mean and standard error of the accumulation rate of TOPRO-stained bacteria. Different letters denote significant differences between treatments using a Tukey test; PAR: photo-synthetically available radiation. * $p < 0.0005$. na: not applicable

Treatment	Accumulation rate of TOPRO + bacteria (d^{-1})			
	July ($df = 3, 7$)		August ($df = 5, 10$)	
UV (0.8 μm)	0.53 $\pm$ 0.08	A	0.41 $\pm$ 0.04	A
Deep (0.8 μm)	0.15 $\pm$ 0.01	B	0.10 $\pm$ 0.01	C
UV (200 μm)	0.50 $\pm$ 0.02	A	0.43 $\pm$ 0.02	A
Deep (200 μm)	0.10 $\pm$ 0.008	B	0.02 $\pm$ 0.01	C
PAR (0.8 μm)	na		0.28 $\pm$ 0.007	B
PAR (200 μm)	na		0.25 $\pm$ 0.01	B
F statistic	63.9*		59.2*	

radiation in the UVA (320 to 400 nm) spectrum (Vincent & Roy 1993). Interestingly, this observed increase in UVB and in the UVB:UVA ratio did not appear to be a function of a decrease in stratospheric ozone levels but was likely a seasonal function of greater solar zenith angles, atmospheric aerosols or differences in tropospheric gas concentrations (Madronich et al. 1995).

The bacterial damage caused by the exposure of natural aquatic communities to solar radiation as demonstrated in this study reinforces and extends observations made in other habitats (Bailey et al. 1983, Herndl et al. 1993, Jeffrey et al. 1996a,b). Bacterial production is often reduced in samples exposed to UVB radiation (Herndl et al. 1993, Helbling et al. 1995, Müller-Niklas et al. 1995, Jeffrey et al. 1996a, Sommaruga et al. 1997, Arrieta et al. 2000, Wilhelm & Smith 2000). Although this reduction in community production has been attributed to cellular DNA damage caused by UVB (Jeffrey et al. 1996b), our results suggest that such cellular damage may be severe enough to result in an increase in membrane permeability and likely cell death.

Although we observed a high proportion of TOPRO+ cells in bags incubated in surface waters, treatments with no light, as well as samples from the ambient lakes waters, consistently had much lower relative numbers of damaged cells. For example, we followed the proportion of TOPRO+ cells daily during the course of the 2 experiments in the ambient lake waters and found this to be remarkably constant, at around 10 to 15%. Other authors have reported similar percentages of presumably damaged or dead bacteria in a wide variety of ecosystems (Heissenberger et al. 1996, Williams et al. 1998, Boulou et al. 1999, del Giorgio & Bouvier 2002), suggesting that bacterial communities are subject to cell mortality independent of grazing or viral infection.

We did not detect any systematic differences in the %TOPRO+ cells in the lake waters between July and August sampling periods, suggesting that the strong effects of shifting UVR levels that we observed in some of the bags must have been greatly modulated in the lakes waters. It is clear that *in situ* bacterioplankton assemblages are generally not exposed to constant subsurface levels of solar irradiation but rather circulate constantly within the epilimnion and experience variable levels of light during a diurnal cycle, so it is not surprising that the %TOPRO cells was on average low in the epilimnion. More interesting perhaps is the constancy of the proportion of TOPRO+ cells in lake waters, which suggests a relatively tight balance between the production and disappearance of damaged cells *in situ*. The fate of the TOPRO+ cells is still unclear and must be further investigated.

An alternative explanation for the increased proportion of TOPRO+ cells in the July experiment may be that the bacterial community at that time was more susceptible to UV inhibition. We are only beginning to understand the diversity of aquatic bacterial communities (Giovannoni & Rappé 2000), but there is now some evidence that certain bacterial groups are more susceptible to UV exposure than others (Joux et al. 1999, Arrieta et al. 2000). Perhaps bacterial species succession over the course of the summer (Pinhassi et al. 1999, Pinhassi & Hagström 2000) resulted in a more UV-resistant community toward the end of August.

Another interesting observation was that the accumulation rate of TOPRO+ bacterial cells was not affected by the presence of grazers and was only influenced by UV exposure (Table 2). This would imply that the nanoflagellate grazers, when present in these treatments, could not effectively remove these damaged cells. The accumulation of TOPRO+ cells could be due in part to a reduction in the rate of nanoflagellate grazing caused by UV exposure (Sommaruga et al. 1996, Mostajir et al. 1999), given that HNF abundance was similar between 200 μm treatments. Another possibility is that the selective grazing by nanoflagellates allowed for a non-grazer effect in the accumulation of damaged cells. HNFs preferentially feed on larger (Bird & Kalff 1993), dividing (Sherr et al. 1992), metabolically active bacterial cells (Gasol et al. 1995, del Giorgio et al. 1996b). Non-selective bacterial grazers, such as filter-feeding cladocerans (Vaque & Pace 1992), absent from our incubations, may play a more important role in removing dead or non-active bacteria than selective flagellate grazers. However, this idea remains to be tested.

Although TOPRO+ bacteria and viral abundance increased as a function of UVR exposure, it is not clear whether there is a link between cell lysis due to viral infection and the loss of membrane integrity as determined by TOPRO staining. It is conceivable that TOPRO may stain the remains of lysed cells, but this would be evidenced as an overall decline in side and forward scatter of TOPRO cytograms relative to SYTO cytograms (used for total cell counts), but this was never the case. It is also possible that at least some TOPRO+ cells are bacteria that are at the end of the lytic cycle, not yet lysed but that are already functionally dead and have lost membrane integrity. This possibility remains to be explored in the future.

Regardless of whether there is a direct connection between the production of TOPRO+ cells and viruses, both components seemed to respond to UVR. The increase in the number of viruses observed in the surface exposed treatments (Fig. 4A,B) suggests that viral production was stimulated in the presence of UV. We hypothesize that the exposure of this bacterial

community to solar radiation with intense ambient UV resulted in the induction of the lytic pathway in lysogenic bacteria. Ambient radiation conditions appeared to be strong enough to induce that response in both experiments, suggesting that damage was severe enough to turn on the error-prone SOS repair mechanism (Freifelder 1987). Indeed, recent evidence has shown the activation of the *recA* gene in natural aquatic bacterial communities exposed to ambient UVR (Booth et al. 2001). RecA, an enzyme produced when the SOS response is activated, cleaves the phage repressor protein in lysogenic bacteria. This results in lytic phage expression, host cell lysis and release of progeny phage (Neidhardt et al. 1996).

Two alternative explanations could also explain the observed increase in viral abundance in the surface treatments. One would be that the increase in labile dissolved organic matter (DOM) photodegradation products resulted in an increase in the bacterial growth rate, thus enhancing viral lytic activity. Total bacterial community growth rates were indeed higher in the surface samples and in the grazer-free treatments. However, when we corrected for TOPRO+ cells, as we assumed these cells were too damaged to act as viable viral hosts, there was no significant change in community growth rates between the surface and deep treatments. Growth did, however, remain higher in the grazer-free treatments. Interestingly, viruses exposed to UV have also been shown to lose their infectivity (Suttle & Chen 1992). This would further impinge on any enhanced viral lytic activity in these surface treatments. Another possible explanation is that exposure to solar radiation resulted in a change in bacterial community composition, wherein the community shifted toward fewer species that were more UV tolerant. A reduction in the number of species might make the community more vulnerable to specific viral attack, again resulting in enhanced lytic viral activity. However, the observed increase in the number of viruses in this study occurred quite rapidly, likely before a major shift in community composition was observed.

Of all the possible explanations to describe the observed increase in viral abundance, the induction of lysogenic bacteria seems the most plausible. Although we did not measure lysogeny directly, the rate of induced viral lysis in lysogenic bacteria caused by exposure to solar radiation could be inferred from the difference in bacterial mortality caused by viral lysis in the surface and the deep treatments. Deep treatments would take into account additional viral production brought about from other treatment causes, and the difference in production between surface and deep treatments would be the production caused by exposure to solar radiation. From the total number of viruses produced, the number of virally lysed bacteria

could be estimated assuming a burst size of 30 viruses per bacterium (Jiang & Paul 1996, Weinbauer & Suttle 1996). This estimate of the number of lysed bacteria divided by the average bacterial abundance and the incubation time would give us an estimate of induced viral mortality per day.

We estimated an induced viral mortality rate of 12.6 % d⁻¹ in the non-grazer treatment and 4.6 % d⁻¹ in the grazer treatment in July, and 8.9 and 4.7 % d⁻¹ in August treatments, respectively. Interestingly, the induced viral rate did not differ significantly between experiments in their respective surface treatments, suggesting that even low ambient levels of UVB are stressful enough to result in the lytic induction of lysogenic cells. However, the presence of grazers seemed to strongly influenced this rate. In both experiments, the estimated induced viral mortality was reduced by approximately 50 % in the presence of nanoflagellate grazers. This indicates that grazers may play a significant role in virus removal, either directly via ingestion or indirectly via ingestion of bacterial hosts. The direct removal of viruses via flagellate grazing has been previously demonstrated to be insignificant for the viruses (Gonzalez & Suttle 1993). The results here indicate that another important virus-grazer interaction occurs via the removal of viruses through flagellate ingestion of infected bacteria.

There is another line of evidence supporting the idea that viruses and HNFs compete for the same bacteria, those bacteria identified as 'metabolically active'. In this study we observed that the VBR is greater in the absence of grazers. As noted above, grazers of bacteria feed preferentially on larger, metabolically active bacterial cells. Viruses are obligate parasites and, in order for an infection to be successful, the virus must replicate in an active host (Ackerman & Dubow 1987). Compared to the rest of the bacterial community, active cells tend to be larger (Gasol et al. 1995, del Giorgio et al. 1996b). Weinbauer & Höfle (1998) found that the smallest bacterial cells in the Plußsee were least often infected, supporting this argument. However, they also found that the very largest cells in the epilimnion were rarely infected. These largest cells represented 10 % of the community but 25 % of the biomass; they are clearly qualitatively different from the bulk of the bacterial community and may be predation-resistant bacteria (Jürgens & Güde 1994). Therefore, the more abundant size class of 0.6 to 1.2 µm bacteria, the size class most suitable to protistan grazers, is what these 2 important predators of bacteria are probably competing for. Indeed, as Binder (1999) pointed out in a mathematical model, if the grazing of infected cells is not taken into account, then the bacterial mortality caused by viral infection would be overestimated.

To sum up, this study has shown that solar radiation results in loss of bacterial membrane integrity, presumably leading to cell damage and perhaps even death. Using the exclusion nucleic acid stain TOPRO, we measured a dramatic increase in the rate of production of damaged cells as a direct result of increasing ambient UVB intensity and exposure to solar radiation. A proportionately higher rate of damage caused by exposure to solar radiation was observed when the ambient UVB conditions were more intense. We also recorded large increases in viral density with increasing exposure to UV, and we hypothesize that this increase was at least partly due to bacterial mortality associated with the UV induction of the lytic cycle. Our results suggest that the proportion of lysogens in the community may greatly influence how UVR affects the dynamics of bacterioplankton assemblages. Our results further suggest that bacterial mortality due to viral infection and protozoan grazing are not independent because bacteriophages and protozoans appear to be competitors for the same bacterial prey.

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