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Single-cell Gel/Comet Assay Applied to the Analysis of UV Radiation–induced DNA Damage in *Rhodomonas* sp. (Cryptophyta)¶

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ABSTRACT

The single-cell gel/comet assay is an electrophoretic technique used to detect single-strand breaks in DNA. Damage is assessed examining individual cells under an epifluorescent microscope. UV-induced DNA damage consists mostly of the formation of pyrimidine dimers; therefore, most of the damage cannot be detected using a standard comet assay. The enzyme T4 endonuclease V breaks DNA strands at sites of pyrimidine dimers. The main objective of this work is to evaluate the comet assay to detect UV-induced damage in DNA after an initial treatment of cells with T4 endonuclease V. This work was conducted on *Rhodomonas* sp. (Cryptophyta), a marine unicellular flagellate. Cells of *Rhodomonas* sp. were exposed to 12 h visible + ultraviolet-A + ultraviolet-B (VIS + UVA + UVB) and VIS (control), with and without T4 endonuclease V. Cells exposed to VIS + UVA + UVB showed approximately 200% more damage than control if these were treated with T4 endonuclease V. *Rhodomonas* sp. were exposed to 3, 6, 9 and 12 h of VIS, VIS + UVA and VIS + UVA + UVB. Damage induced by VIS + UVA + UVB as detected by the comet assay increased along with exposure time. However, damage caused by VIS and VIS + UVA remained relatively constant at all times. Results of this study indicate that the comet assay is more sensitive to UV radiation damage when used in conjunction with T4 endonuclease V. This modification of the comet assay can be used as an alternative technique to detect DNA damage in single cells caused by UV radiation.

INTRODUCTION

As predicted in the mid-1970s the stratospheric ozone layer has diminished as a consequence of chlorofluorocarbons produced by human activity (1,2). The thinning of the ozone results in increased surface flux of harmful ultraviolet-B (UVB, 280–320 nm)† radiation relative to ultraviolet-A (UVA, 320–400 nm) and visible (VIS, 400–700 nm) radiations (3–5). The greatest relative ozone loss (>50% compared to normal conditions) occurs at high latitudes in both hemispheres during late winter and early spring (6). Even under ozone-depleted conditions UVA, UVB and VIS radiations in Antarctic waters are much lower than in tropical and temperate latitudes (7).

DNA structural damage produced by UV radiation is diverse (8). Nucleic acid bases absorption spectrum peaks around 260 nm in the ultraviolet-C (UVC) range and its tail extends well into the UVB range (9). The cyclobutane pyrimidine dimer is the predominant photoproduct induced by UVB along with the pyrimidine (6-4) photoproduct and its photoisomer, the Dewar pyrimidinone (9,10). Dimerization results in mutation and causes the RNA polymerase to stall during transcription (9). It possibly leads to cell cycle arrest and reduction in the growth rate of microalgae (11).

UVA-induced DNA damage mechanisms are poorly understood (12). UVA has very low induction rates and produces damage indirectly through oxygen and hydroxyl radicals, highly reactive chemical intermediates (13) which react with DNA to form monomeric damage (photohydrates), strand breaks and DNA crosslinks (8).

The alkaline version of the single-cell gel (SCG)/comet assay is a sensitive electrophoretic technique (14,15) that can be used to detect single- or double-strand breaks in DNA of single cells (16). In this technique cells are embedded in agarose gel and placed on a microscope slide. The cells are lysed and the liberated DNA is electrophoresed under alkaline (pH 12.3 or >13) conditions. After staining with a suitable stain and viewed by epifluorescence microscopy, cells with strand breaks resemble a comet, where broken strands have migrated from a brightly fluorescent core (or nucleoid) toward the anode. When cells are electrophoresed under alkaline condition single- and double-strand breaks, and alkalilabile lesions in the DNA can be detected. The distance and/or amount of migration are indicative of the number of strand breaks (16,17).

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†*Abbreviations:* BSA, bovine serum albumin; EDTA, ethylenediamine-tetraacetic acid; PER, photoenzymatic repair; SCG, single-cell gel; UVA, ultraviolet-A; UVB, ultraviolet-B; UVC, ultraviolet-C; VIS, visible.

An approach for detecting UV radiation–induced base damage has been introduced by Collins *et al.* (18) using the SCG/comet assay electrophoresed under alkaline (pH > 13) conditions. They demonstrated that DNA damage caused by UVC radiation in rodent mutant cell lines could be probed for pyrimidine dimers after the lysis step, incubating embedded nuclei with T4 endonuclease V, which breaks DNA strands at sites of pyrimidine dimers (19,20). UV-induced DNA damage in unicellular algae can be detected at the cellular/organism level if the comet assay is combined with an initial incubation with T4 endonuclease V. Thus variability in damage among cells can be detected.

Our work was performed on the unicellular marine phytoflagellate *Rhodomonas* sp. (Cryptophyta). Like other marine flagellates the cells of *Rhodomonas* sp. are found in most environments and are ''naked,'' meaning they lack a cell wall (21). The main objective of this study was to evaluate the use of the SCG/comet assay to detect UV radiation–induced DNA damage in phytoplankton, to adapt it to be used in the field to detect single-cell damage. A necessary step was to evaluate the effect of using T4 endonuclease V to enhance the sensitivity of this test to UV radiation.

MATERIALS AND METHODS

Cell culture. Clonal cultures of *Rhodomonas* sp. (Cryptophyta) #768 were initiated from axenic cultures obtained from Provasoli–Guillard National Center for Culture of Marine Phytoplankton (CCMP, West Boothbay Harbour, ME). Cells were initially collected from New Zealand in 1983 and deposited in CCMP in 1984. Cell width ranged from 9 to 13 μm (22). Cells were maintained in 125 mL Erlenmeyer flasks containing approximately 60 mL of f/2-Si medium (Sigma–Aldrich Corporation, St. Louis, MO). Cultures were incubated at 19–21°C on a 12:12 light–dark cycle under a VIS irradiance of 7.12 W/m^2. Cultures used for experimentation were in log phase as determined by changes in *in vivo* fluorescence. These were grown in 35 cc capacity quartz test tubes (GM Associates, Inc., Oakland, CA).

Experiments were performed inside a wooden box 1.22 × 0.91 × 0.61 m, painted with nontoxic, flat, nonreflective black paint, containing VIS, UVA and UVB sources (23). The box was kept inside a walk-in incubator at Scripps Institution of Oceanography, La Jolla, CA. An air extractor fan was mounted on one end of the box and an intake vent on the other end; the airflow maintained a constant air temperature of 18°C during all experiments. VIS radiation was provided by two 48 in. Sylvania Supersaver Cool White F40CW/SS Rapid Start 35 W fluorescent lamps. UVA radiation was provided by a 48 in. Light Sources F40T12-BL 40 W lamp and UVB by a 48 in. Light Sources F40T12-UVB 40 W lamp. UV lamps were preburned to ensure stable emissions. UV lamps were fixed horizontally, 4 cm apart, in the incubator. The UVB lamp was always covered with cellulose III acetate filter (0.003 in. thickness) to filter radiation below 290 nm (UVC); this was replaced after each experiment. For experimental treatments involving VIS + UVA radiation (>320 nm) a mylar filter (0.003 in. thickness) was placed over the experimental culture. For VIS treatments (control) a 0.30 cm thick Acrylite® OP-3 acrylic filter (filters 100% below 390 nm, 98% below 400 nm) was placed over the control culture in order to filter UVA and UVB radiation. The effectiveness of all filters to block UV radiation was tested before and after each experiment by looking at the % transmittance of the filters using a UV–VIS scanning spectrophotometer. In order to simulate sunlight spectral qualities the UVB lamp was covered with six layers of gray fiberglass screen wire and the UVA lamp with two layers. The irradiance spectra of the incubator using VIS, UVA and UVB lamps was determined using an Optronics Laboratories, Inc. (Orlando, FL) Model 752 spectroradiometer as measured in the black wooden box (Fig. 1). The distance from the sensor to the center of the VIS lamps was 24 cm, and to the center of the UV lamps was 30 cm. Irradiance levels were: VIS, 7.12 × 10^{-4}; UVA, 9.47 × 10^{-5} and UVB, 3.89 × 10^{-6} W/cm^2.

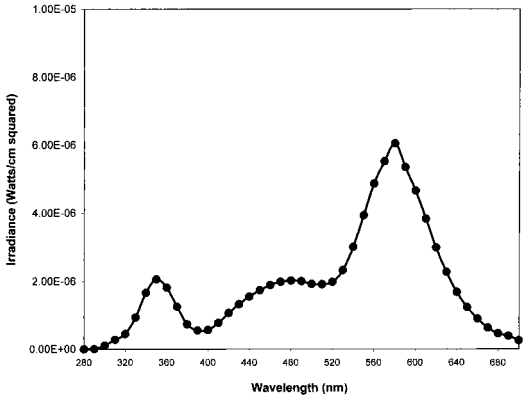

Figure 1. Spectral UV and PAR conditions (in W cm^2/nm) in the culture chamber with cellulose acetate filter, but without mylar or Acrylite® OP-3. Irradiance was measured each 10 nm, from 280 to 700 nm.

Cultures were placed approximately 24 cm from the center of both lamps.

The purpose of using the UV lamps was not to simulate and predict effects caused by solar radiation, but to have controlled radiation conditions to test the comet assay in *Rhodomonas* sp. under a radiation spectrum which most closely resembles UVB, UVA and VIS energy proportions of sunlight, given the limitations of our system.

The SCG/comet assay. SCG electrophoresis or comet assay was performed according to a modification of the technique described by Steinert *et al.* (24). *Rhodomonas* sp. cell suspensions were spun down in a microcentrifuge at 2000 ***g*** for 2 min. The supernatant was discarded and the pellet resuspended in 50–400 μL low melting temperature DNA agarose in Kenney's salt solution (KLMA) (0.65% Fisher Biotech low melting temperature DNA grade agarose in Kenney's salt solution, consisting of 0.4 *M* NaCl, 9 m*M* KCl, 0.7 m*M* K_2HPO_4 and 2 m*M* $NaHCO_3$, pH 7.5) at 36°C. The volume of KLMA depended on cell density, larger pellets being resuspended in larger volumes. Fifty microliters of the cell suspension was transferred on slides previously coated with 0.65% normal melting temperature agarose (Fisher Biotech, Pittsburgh, PA, low electroendosmosis (EEO) agarose) in 40 m*M* Tris–acetate, 1 m*M* ethylenediamine-tetraacetic acid (EDTA), pH 7.5 (TAE), allowed to gel on a stainless steel tray placed over ice and top-coated with 50 μL KLMA. Slides were placed in Coplin jars filled with lysing solution, 2.5 *M* NaCl, 10 m*M* Tris, 0.1 *M* EDTA, 1% Triton X-100 and 10% dimethyl sulfoxide, pH 10.0, and incubated at 4°C for at least 2 h.

After lysis, slides were washed three times for 2 min in Coplin jars filled with bovine serum albumin (BSA) buffer (10% BSA, 0.05 *M* Tris–HCl, pH 7.6, 0.001 *M* EDTA, 0.05 *M* NaCl, 0.001 *M* dithiothreitol) (20) and drained. Fifty microliters of buffer (control treatment) or T4 endonuclease V (4 U/slide) (experimental treatments) in buffer were transferred to slides. These were sealed with a cover glass and incubated for 30 min at 37°C. The cover glass was then removed by immersing the slides in distilled water.

Slides were transferred to Coplin jars filled with distilled water and washed three times for 2 min in order to remove excess salts. Slides were then placed in a submarine-gel electrophoresis chamber filled with alkaline electrophoresis buffer (300 m*M* NaOH, 1 m*M* EDTA) and DNA were allowed to unwind for 15 min; after which electrophoresis was performed at a constant voltage of 12 V, ≈185 mA, for 60 min. The slides were neutralized by three 2 min rinses in 0.4 *M* Tris, pH 7.5 and dehydrated by a 5 min rinse in cold 95% ethanol. Slides were dried at 27°C and stained with 40 μL ethidium bromide (20 μg/mL).

The slides were examined under a Nikon Optiphot microscope with epifluorescent set (excitation filter 510–560 nm green light, barrier filter 590 nm) at 200× magnification. Comet parameters were measured and analyzed using a Komet® Image Analysis System software version 3.1 (Integrated Laboratory Systems, Research Triangle Park, NC and Kinetic Imaging, Ltd., Liverpool, UK) (25). Each slide

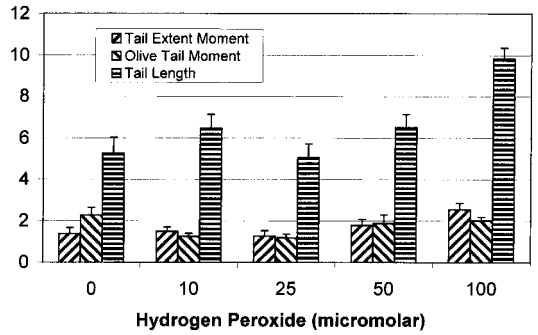

Figure 2. DNA damage in the hydrogen peroxide exposure experiments using 30 min alkaline electrophoresis. Damage expressed in tail extent moment, Olive tail moment and tail length. Vertical lines: +1 standard error of mean.

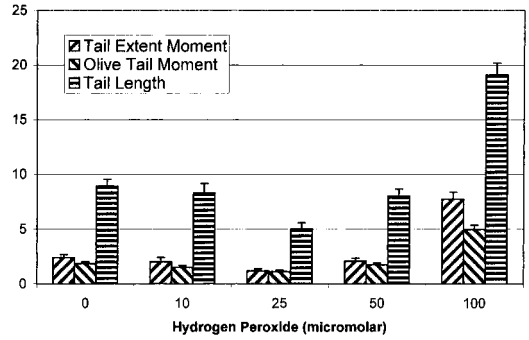

Figure 3. DNA damage in the hydrogen peroxide exposure experiments using 60 min alkaline electrophoresis. Damage expressed in tail extent moment, Olive tail moment and tail length. Vertical lines: +1 standard error of mean.

was divided into five regions; in each region at least 10 cells were randomly selected and analyzed (approximately 50 cells/slide).

Hydrogen peroxide exposure experiments. These experiments were performed in order to preliminarily examine the effectiveness of the comet assay on *Rhodomonas* sp. in terms of overall cell sensitivity to DNA damage and to examine the shape and size of damaged and undamaged nucleoids. Cell suspensions were spun in a microcentrifuge at 2000 ***g*** for 2 min. The supernatant was decanted and the pellet resuspended in hydrogen peroxide dilutions (explained below) for 15 min, placing the microcentrifuge tubes on ice. Suspensions were spun as before and washed with filtered seawater. Cells were spun again and rewashed before performing the comet assay.

Hydrogen peroxide concentrations of 0, 10, 25, 50 and 100 μM were used for these experiments. All dilutions were prepared using filtered f/2-Si medium.

Twelve hours UV exposure T4 endonuclease V experiment. This experiment was performed in order to test if the sensitivity of the SCG/comet assay to UV radiation could be enhanced by an initial incubation with T4 endonuclease V + buffer. Cultures of *Rhodomonas* sp. at exponential growth phase were transferred from 125 mL Erlenmeyer flasks to 35 mL quartz tubes. Two quartz culture tubes were closed with Teflon stoppers modified with a glass L-shaped air vent and submerged 2 cm under distilled water (to avoid leakage of water into the tubes) in order to avoid excessive heating. One tube was exposed to continuous 12 h VIS + UVA + UVB (experimental treatment) radiation in the incubator described above. Another was exposed to the same conditions but in addition an Acrylite® OP-3 acrylic filter was placed horizontally over the culture tube in order to block all UV radiation (control treatment) (Fig. 2). The comet assay was performed on duplicate slides, with and without the addition of T4 endonuclease V, after the initial incubation and after 12 h in experimental and control treatments. The comet assay was performed in cultured cells, with and without the initial incubation with T4 endonuclease V, and also in cells exposed to the 12 h experimental and control treatments, with and without the incubation with the enzyme.

Twelve hours UVA/UVB dose experiment. By means of this experiment we attempt to separate the effects of UVA and UVB radiation on a 12 h dose experiment. Cultures of *Rhodomonas* sp. at exponential growth phase were transferred from 125 mL Erlenmeyer flasks to 35 mL quartz tubes. Before the start of the experiment (time = 0) the comet assay was performed in duplicate slides on cells from the original culture. Three quartz tubes were set inside the culture chamber as described above. An Acrylite® OP-3 filter was placed horizontally over one culture tube and a Mylar filter over another, blocking UVA + UVB and UVB, respectively. The third tube was left uncovered, therefore exposed to VIS + UVA + UVB. Culture samples from each tube were aseptically taken after 3, 6, 9 and 12 h after the start of exposure. The comet assay was performed after an initial incubation with T4 endonuclease V + buffer, at each exposure time, on duplicate slides for each treatment.

Statistical analysis. One-way analysis of variance (ANOVA) was used to test for differences between hydrogen peroxide concentrations, and between slides treated and untreated with T4 endonuclease V, in the control and experimental treatments, using tail extent moment, Olive tail moment and tail length as dependent variables. This test was also used in the 12 h UVA/UVB dose experiment to test for differences between time exposures within each treatment, using tail extent moment as the dependent variable (26). Fisher's protected least significant difference (PLSD) tests were used to compare between pairs of means after performing ANOVA. Whenever necessary data were transformed with ln X or ln X + 1 in order to fulfill assumptions of homoscedasticity. Statistical analyses were performed using StatView version 4,57,0,0 statistical program (27,28). Any statistical probability ≤ 0.05 was considered significant.

RESULTS AND DISCUSSION

Hydrogen peroxide exposure experiments

Figures 2 and 3 show levels of DNA damage in *Rhodomonas* sp. cells exposed to different concentrations of hydrogen peroxide. Damage is expressed in terms of ''tail length,'' which measures the distance (in pixel units) of migrating DNA from the nucleus toward the anode in the electrophoresis tray; ''tail extent moment'' (tail length × tail %DNA) and ''Olive tail moment'' ([tail profile center of gravity − head profile center of gravity] × tail %DNA) (25). Cells exposed to 60 min electrophoresis showed greater damage at all hydrogen peroxide concentrations than when exposed to 30 min in terms of tail extent moment, Olive tail moment and tail length. Lower *P* values in ANOVA, indicating greater significant differences, were also obtained for most measured parameters for the 60 min electrophoresis (Table 1). Significant differences between damage observed at 100 μM and most other H_2O_2 concentrations were observed, specially for the 60 min electrophoresis. Since higher sensitivity was achieved by electrophoresing cells for 60 min, this time interval was used in all subsequent electrophoresis. Hydrogen peroxide causes single-strand breaks, double-strand breaks as well as altered bases. Damage is done through the formation of reactive oxygen species such as superoxide and hydroxyl radicals (29).

Twelve hours UV exposure T4 endonuclease V experiment

Figure 4 shows levels of DNA damage in cells after 12 h exposure to VIS + UVA + UVB (experimental exposure treatments) or VIS alone (control exposure treatments); with (experimental enzyme treatments) and without (control en-

Table 1. Analyses of variance to test for differences between effects in experiments

	DF*	Mean square	*F*-value	*P*-value
Hydrogen peroxide experiment, 30 min., Effect: hydrogen peroxide concentration				
Tail extent moment (ln)†	4; 245	1.954; 0.391	4.993	0.0007
Olive tail moment (ln)	4; 245	1.173; 0.289	4.058	0.0033
Tail length (ln)	4; 245	6.284; 0.802	7.832	<0.0001
Hydrogen peroxide experiment, 60 min., Effect: hydrogen peroxide concentration				
Tail extent moment (ln)	4; 245	14.891; 0.432	34.432	<0.0001
Olive tail moment (ln)	4; 245	8.466; 0.227	37.218	<0.0001
Tail length (ln)	4; 245	15.012; 0.632	23.756	<0.0001
12 h UV Endonuclease V experiment. Effect: enzyme/UV treatment				
Tail extent moment (ln)	3; 196	18.824; 0.403	46.677	<0.0001
Olive tail moment (ln)	3; 196	12.084; 0.193	62.613	<0.0001
Tail length (ln)	3; 196	0.269; 0.074	3.627	0.0140
12 h UV-A/UV-B dose experiment., Effect: exposure time				
PAR (ln)	4; 210	1.821; 0.984	1.851	0.1203
PAR + UV-A (ln)	4; 245	4.250; 1.044	4.072	0.0033
PAE + UV-A + UV-B (ln)	4; 210	10.838; 0.937	11.562	<0.0001

*DF, degrees of freedom; *P*-value, probability.
†Data transformed with natural logarithm (ln).

zyme treatments) the initial incubation with T4 endonuclease V. ANOVA indicates highly significant differences ($P < 0.0001$) among treatments for all measured parameters (Table 1). Measures of tail extent moment and Olive tail moment indicate cultures exposed to VIS + UV radiation have significantly higher levels of damage than those exposed to VIS alone; cells treated with T4 endonuclease V showed greater damage to UV radiation (Fig. 4, Table 2). Among all reported ''comet'' parameters greater damage by UV radiation (in terms of fold increase) was detected using tail extent moment.

Incubation with T4 endonuclease V enhanced UV radiation–induced damage levels as measured by tail extent moment or Olive tail moment. Both parameters take into consideration %DNA measured as fluorescent intensity. According to Gedik *et al.* (30) an increase in fluorescent intensity of the comet tail rather than its length is the predominant effect of increasing DNA break frequency; which is the predicted consequence of the relaxation of more and more loops of DNA by strand breaks. Endonuclease III specifically nicks DNA at sites of oxidized pyrimidines (31) while T4 endonuclease V nicks at sites of pyrimidine dimer formation (19,20). These types of damage are detected by the comet assay as single-strand breaks; therefore the sensitivity of the test is increased. The sensitivity of the comet assay to UV radiation has been greatly enhanced in human HeLa cells using T4 endonuclease V, extending the detection of this to less than 0.1 break per 10^9 Da of DNA (19). Endonuclease III has been used to enhance the sensitivity of the comet assay in human HeLa cells and lymphocytes treated with H_2O_2 (32).

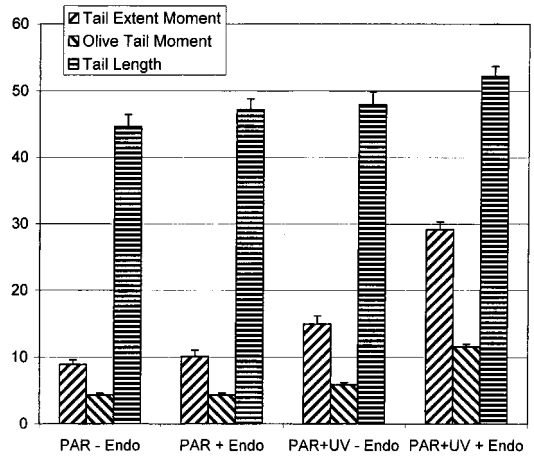

Figure 4. DNA damage in the 12 h UV exposure experiments, with and without T4 endonuclease V. PAR, PAR radiation only; PAR + UV, PAR + UVA + UVB radiation; +Endo, with T4 endonuclease V; −Endo, without T4 endonuclease V. Damage expressed in tail extent moment, Olive tail moment and tail length. Vertical lines: +1 standard error of mean.

Twelve hours UVA/UVB dose experiment

Figure 5 shows DNA damage in tail extent moment, which according to the previous experiment detects DNA damage of cells exposed to VIS + UV radiation. *Rhodomonas* sp. cells were exposed to 0, 3, 6, 9 and 12 h VIS, VIS + UVA and VIS + UVA + UVB radiation (0, 10.2, 20.5, 30.7 and 40.9 kJ/m^2 UVA dose; 0, 0.4, 0.8, 1.3 and 1.7 kJ/m^2 UVB dose). Cells were incubated with T4 endonuclease V in all treatments prior to performing the comet assay in order to increase the sensitivity. DNA damage in cells exposed to VIS did not differ significantly at any exposure time (Table 1). However, significant differences were observed in tail extent moment in cells exposed to VIS + UVA between 0 and 3, 6, 9 and 12 h (Fisher's PLSD, $P \leq 0.0142$). Significant differences were also observed between all exposure times (except 0 *vs* 3, 3 *vs* 6 and 6 *vs* 9 h) in cells exposed to VIS + UVA + UVB radiation (Fisher's PLSD, $P \leq 0.0346$); DNA damage levels increased significantly with exposure time (ANOVA: degrees of freedom = 1, 3; $F = 45.726$; $P = 0.0066$) ($R^2 = 0.938$).

The damage induced by VIS + UVA between 3 and 12 h was not significant or was repaired simultaneously within

Table 2. Fisher's PLSD comparison among all pairs of means for treatments in the 12 h exposure T4 endonuclease V experiment. PAR, PAR radiation only; PAR + UV, PAR + UV-A + UV-B radiation; +Endo, with T4 endonuclease V; −Endo, without T4 endonuclease V. Damage expressed in tail extent moment, Olive tail moment and tail length

Comparison	Tail extent moment		Olive tail moment		Tail length	
	Mean difference	*P*-value	Mean difference	*P*-value	Mean difference	*P*-value
PAR/−Endo V *vs* PAR/+Endo V	−0.077	0.5455	0.015	0.8608	−0.070	0.2022
PAR/−Endo V *vs* PAR/ + UV/−Endo V	−0.516	<0.0001	−0.275	0.0020	−0.075	0.1710
PAR/−Endo V *vs* PAR/ + UV/+Endo V	−1.337	<0.0001	−1.033	<0.0001	−0.178	0.0013
PAR/+Endo V *vs* PAR/ + UV/−Endo V	−0.439	0.0007	−0.290	0.0011	−0.005	0.9247
PAR/+Endo V *vs* PAR/ + UV/+Endo V	−1.261	<0.0001	−1.048	<0.0001	−0.108	0.0481
PAR + UV/−Endo V *vs* PAR + UV/+Endo V	−0.821	<0.0001	−0.758	<0.0001	−0.103	0.0597

the course of the experiment. DNA repair could have been possible by photoreactivation which is brought about by light with wavelengths between 330 and 450 nm (regions in VIS and UVA bands) (32) or by other repair mechanism such as nucleotide excision repair (33).

Net DNA damage induced by VIS + UVA + UVB radiation accumulated over time, indicating damage levels were higher than repair levels. Since DNA absorbs in the lower portion of the UVB band (9) the increase in damage over time was to be expected. This experiment shows the harmful effects of UVB radiation on *Rhodomonas* sp. growing under culture conditions. UVA and UVB radiation levels used in this experiment have been reported to occur in the water column at mid latitudes (34). In the ocean planktonic unicellular algae are subject to vertical and horizontal migrations and could therefore have decreased or increased levels of DNA damage (35,36).

UVB radiation generates direct induction of damage to form photoproducts such as thymine–thymine cyclobutane dimers, which occurs with greatest frequency; UVA generates oxygen and hydroxyl radicals which react with DNA to form monomeric damage, strand breaks and DNA–protein crosslinks (8). At the same time wavelengths in the UVA–lower visible region promote photoenzymatic repair (PER). The wavelength dependence of PER efficiency is variable between species (8,37). Our results suggest DNA damage induced by UVA from 3 to 12 h was compensated by PER and other types of repair, but repair mechanisms could not neutralize further damage induced by adding UVB to the system.

There is little information published on microalgae about wavelength-related changes in DNA damage, and to our knowledge nothing has been published which measures damage using the SCG/comet assay. Therefore, few comparisons can be made with other studies. However, Buma *et al.* (11) using monoclonal antibodies and flow cytometric analyses found higher damage in the *Cyclotella* sp. (Bacillariophyceae) cells induced by wavelengths corresponding to the UVB band (as expected) and very little or no damage induced by UVA, as was observed in this study (Fig. 5).

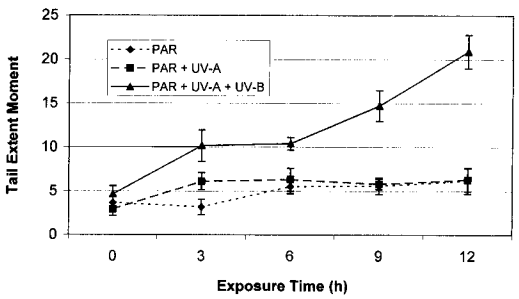

Figure 5. DNA damage expressed in tail extent moment during the 12 h dose experiment for cultures treated with PAR, PAR + UVA and PAR + UVA + UVB radiation. Vertical lines: ±1 standard error of mean.

The nearly linear relationship observed in this study between exposure time (VIS + UVA + UVB) and tail extent moment (R^2 = 0.938) shows a dose-dependent increase in DNA damage in *Rhodomonas* sp. (Fig. 5). A UVB dose-dependent increase in DNA damage was also observed by Gieskes and Buma (38) in *Emiliana huxleyi* (Prymnesiophyceae), where they imply an acceleration of the effects with higher doses (using biologically effective daily doses).

The SCG/comet assay with the modifications described here provides an alternative method to measure UV radiation–induced DNA damage in microalgae. This technique has several advantages: (1) data are collected at the level of the individual cell, providing, in unicellular microalgae, information on average damage levels as well as variability in damage between individual organisms; (2) only a few thousand cells are required per slide preparation, therefore experiments can be performed using low-volume cultures; (3) it is relatively simple to perform as compared with other methods used to assess DNA damage since DNA extraction is not needed (as in the alkaline elution method); and (4) it is relatively inexpensive to realize (16).

For the comet assay described in this paper it is advisable to use an image analysis system capable of analyzing %DNA in tail (or similar parameter) since it is more sensitive to UV-induced DNA damage, as can be observed in Fig. 4. The enzyme T4 endonuclease V, which is essential to increase the sensitivity of this assay, is currently commercially available (Epicentre Technologies), making this test available to a wider number of people.

The comet assay could be potentially used with field samples under some circumstances. In most common situations where a multispecies phytoplankton assemblage is observed it might be too difficult to differentiate damage between species, or even taxonomic groups under the epifluorescent microscope. However, it could be used successfully in cases where phytoplankton composition is dominated by one or few species, such as in some phytoplankton blooms or in

some epiphytic algae. The comet assay can also be used in microalgae for genotoxicity assessment induced by pollutants or other parameters which directly or indirectly (through the formation of oxygen free radicals) damage DNA, such as heavy metals and hydrocarbons (39).

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