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A Simple and Efficient Method for the Quantitative Analysis of Thymine Dimers in Cyanobacteria, Phytoplankton and Macroalgae

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Summary. Cyclobutane dimers are the most common DNA lesions after exposure of cells to UV-B radiation. A quantitative method was developed to determine the frequency of thymine dimers in aquatic primary producers such as cyanobacteria, phytoplankton and macroalgae to study the effects of UV radiation. Genomic DNA was extracted and purified by using standard biochemical and molecular biology techniques. DNA was transferred to a nylon membrane in a slot or dot blot and incubated with a primary antibody (anti thymine dimer KTM53) against thymine dimers. The secondary antibody was an anti-mouse IgG (Fab specific) peroxidase conjugate. The blots were quantified in a Kodak Digital Science Image Station 440 CF by the chemiluminescence method. The calibration of the method was achieved by using the plasmid pBSK with known DNA sequence, length and number of adjacent thymine pairs. This method permits the measurement of low as well as high levels of DNA lesions in nanogram quantities of DNA. This method can be used for cultured as well as naturally occurring organisms.

Key words: antibody, chemiluminescence, cyanobacteria, macroalgae, phytoplankton, thymine dimers, UV radiation.

INTRODUCTION

Ultraviolet radiation induces deleterious effects in all living organisms ranging from prokaryotic bacteria and unicellular aquatic organisms to higher plants, animals and men. While UV-C (<280 nm) radiation is ecologically not relevant since it is quantitatively absorbed by oxygen and ozone in the Earth's atmosphere, the longer wavelength UV-B (280-315 nm) and UV-A (315-400 nm) radiation can have significant effects on the biota, even though the majority of the extraterrestrial UV-B is absorbed by stratospheric ozone (Madronich *et al.* 1998). Some of the biological effects of solar UV radiation include killing of bacteria (Herndl 1997), inhibition of motility and orientation, protein destruction, pigment bleaching and photoinhibition of photosynthesis in cyanobacteria, phytoplankton and macroalgae (Cullen *et al.* 1992; Arrigo 1994; Sinha *et al.* 1995, 2001; Sinha and Häder 1996; Häder *et al.* 1998; Neale *et al.* 1998) as well as lethality in primary and secondary consumers in aquatic ecosystems (Hunter *et al.* 1982, Little and Fabacher 1994). In higher plants growth of leaves, shoots and roots are affected (Bornman and Teramura 1993, Ros and Tevini 1995, Huang *et al.* 1997) and flowering and reproduction are impaired (Staxén and Bornman 1994, Klaper *et al.* 1996) by UV stress.

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Action spectra have been measured for a number of UV-B effects in many organisms and for very different responses (Häder and Liu 1990, Häder *et al.* 1991, Cullen *et al.* 1992) indicating a multitude of cellular targets for solar UV. Proteins strongly absorb around 280 nm, due to their aromatic amino acids. In the photosynthetic apparatus several targets of UV-B radiation have been identified including the water splitting site and the D1/D2 protein complex in photosystem II (Bhattacharjee and David 1987, Renger *et al.* 1989). The integrity of the membranes is affected, caused by a decrease in the lipid content (Murphy 1983).

Photodynamic reactions are potential mechanisms by which ultraviolet radiation damages living cells (Ito 1983). The high energy of short wavelength photons absorbed by chromophore molecules can lead to the formation of singlet oxygen or free radicals known to destroy membranes and other cellular components (Benson *et al.* 1992, Alscher *et al.* 1997, Mackerness *et al.* 1999).

The DNA is certainly one of the key targets for damaging UV-B radiation in bacteria (Peak and Peak 1982, Peak *et al.* 1984), phytoplankton (Buma *et al.* 1995, 1997; Sommaruga and Buma 2000), macroalgae (Pakker *et al.* 2000), plants (Quaite *et al.* 1992), humans and animals (Stein *et al.* 1989, Kripke *et al.* 1992). Radiation damage to DNA is potentially dangerous to cells, since a single photon hit may have a dramatic or even lethal effect. Several different types of DNA damage have been identified that result from free radicals and reactive oxygen species formed by various photochemical processes. The two major classes of mutagenic DNA lesions induced by UV radiation are *cis-syn* cyclobutane-pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) which are pyrimidine adducts (Mitchell and Karentz 1993, Prakash *et al.* 1993, Friedberg *et al.* 1995, Sancar 1996a, Thoma 1999, Lindahl and Wood 2000). Both classes of lesions distort the DNA helix. CPDs and 6-4PPs induce a bend or kink of 7-9° and 44°, respectively. The ability of UV radiation to damage a given base is determined by the flexibility of the DNA. Sequences that facilitate bending and unwinding are favorable sites for damage formation, e.g. CPDs form at higher yields in single-stranded DNA, at the flexible ends of poly(dA).(dT) tracts, but not in their rigid center (Becker and Wang 1989, Lyamichev 1991). Bending of DNA towards the minor groove reduces CPD formation (Pehrson and Cohen 1992). One of the transcription factors having a direct effect on DNA damage formation and repair is the TATA-box binding protein (TBP). TBP promotes the selective formation of 6-4PPs in the TATA-box, where the DNA is bent, but CPDs are formed at the edge of the TATA-box and outside, where DNA is not bent (Aboussekhra and Thoma 1999). These DNA lesions interfere with DNA transcription and replication and can lead to misreadings of the genetic code and cause mutations and death.

In contrast to other DNA lesions, CPDs can be photorepaired by a specific enzyme (photolyase) in the presence of and using the energy of UV-A or visible light at permissive temperatures (Sancar 1996b). Photolyases contain FAD as a catalytic cofactor and a second chromophore as a light-harvesting antenna. The second chromophores are either 5,10-methenyl- tetrahydrofolate or 8-hydroxy-5-deazariboflavin, with absorption maxima of ~380 and ~440 nm, respectively. Other DNA repair systems can also operate without light (Britt 1996, Taylor *et al.* 1996). While many studies have been conducted under laboratory conditions, DNA lesions in intact plants and bacteria have also been measured under field conditions (Quaite *et al.* 1992, Ballaré *et al.* 1996, Jeffrey *et al.* 1996). Even though there is an effective repair of DNA damage (Stapleton *et al.* 1997), some lesions may persist; low temperatures or darkness can hamper the enzymatic repair of DNA damage (Britt 1996, Takeuchi *et al.* 1996).

The aim of this work was to develop a reliable quantitative method to determine the frequency of thymine dimers in aquatic primary producers.

MATERIALS AND METHODS

Organisms and culture conditions

A number of cyanobacteria such as *Anabaena* sp., *Nostoc* sp. and *Scytonema* sp., phytoplankton such as *Euglena gracilis* and *Gyrodinium dorsum* and macroalgae such as *Porphyra umbilicalis* and *Ceramium rubrum* were used in the present study. The cyanobacteria were routinely grown in an autoclaved liquid medium as described by Safferman and Morris (1964) at a temperature of 20°C and illuminated with white fluorescent light of 12 Wm^{-2} (for details see Sinha *et al.* 1995). The phytoplankton was grown in F/2 medium (Guillard and Ryther 1962) prepared with artificial sea water (Tropic Marine, Dr. Bienle GmbH, Germany) in cylindrical glass tubes placed in a Kniese apparatus and bubbled with air at 19°C and continuous illumination (35 Wm^{-2} PAR from mixed fluorescence tubes: OSRAM L 36 W/32 Lumilux de luxe warm white de luxe and

Radium NL 36 W/26 Universal white). Macroalgae were grown in artificial sea water (0.33%; Instant Ocean, Sarrebourg, France and Mentor, Ohio, USA) supplemented with nitrate (9.8 µM) and phosphate (3.1 µM) and illuminated with fluorescent light (12 Wm^{-2}) at a temperature of 4°C for a 12 h photoperiod. All experimental materials are being routinely grown in our laboratory since at least seven years.

UV radiation source

Plastic trays containing macroalgae (fresh weight 2 - 3 g) dipped in 1 cm of water as well as liquid cultures (25 - 30 ml) of cyanobacteria and phytoplankton organisms were exposed to UV radiation produced from a transilluminator (TI 312, Bachofer, Reutlingen, Germany) at a distance of 20 cm which resulted in an irradiance of around 10 Wm^{-2} UV. The irradiance of UV was measured with a double monochromator spectroradiometer (OL 754, Optronic Laboratories, Orlando, Florida, USA). At defined time intervals samples were withdrawn and subjected to DNA extraction and blotting to monitor the formation of thymine dimers. DNA extraction was done either immediately after UV exposure, or the samples were kept in formaldehyde (2 %, v/v) in darkness. All experiments were run in triplicates.

Extraction of DNA

Cyanobacterial and phytoplankton organisms were concentrated by centrifugation (J2-21M/E) using a JA 20 rotor (Beckman Instruments) at 500 x g for 10 min at room temperature. All the samples (non irradiated control and UV irradiated) were washed twice with 2 ml of a solution containing 50 mM Tris-HCl, pH 8.0, 5 mM EDTA and 50 mM NaCl and resuspended in a 500 µl solution containing 50 mM Tris-HCl, pH 8.0 and 50 mM EDTA. Thereafter, except for the macroalgae, cells were broken by sonification (20 Watts, Branson Sonifier 450, Ultrasonic Corporation, Danbury, USA) for 3 min on ice. Macroalgae were homogenized with a mortar and pestle. Subsequently, cells were treated with 100 µg/ml of proteinase K. Thereafter, 1 ml of prewarmed (55°C) extraction buffer containing 3 % (w/v) cetyltrimethyl ammonium bromide (CTAB); 1 % (w/v) sarkosyl; 20 mM EDTA; 1.4 M NaCl; 0.1 M Tris-HCl, pH 8.0 and 1 % (v/v) 2-mercaptoethanol were added and incubated at 55°C for 1 h in a water bath with mixing by gentle inversion every 10 min. The resulting suspension was allowed to cool for 1 - 2 min, and thereafter 2 ml of chloroform : isoamyl alcohol (24:1, v/v) was added and mixed by gentle inversion (about 25-30 times) until an emulsion was formed. After centrifugation (12000 x g for 5 min at room temperature) the supernatant was transferred to sterile microcentrifuge tubes. DNA was precipitated at -20°C for 2 h in 2 volumes of ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) and centrifuged at 12000 x g for 30 min at 4°C. The pellet was briefly rinsed once with ice-cold 70 % ethanol, dried and rehydrated with agitation at room temperature in TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). DNA samples were processed either immediately after extraction or kept at 4°C for further analyses. The purity of the DNA was determined spectroscopically (Beckman DU-70). The DNA was considered pure if the ratio between 260 and 280 nm was between 1.8 and 2.0. A ratio below 1.6 is typical for a protein contamination while the ratio above 2.0 is characteristic for an RNA contamination. The DNA concentration was measured in a spectrophotometer (Beckman DU-70). The absorption at 260 nm gives the concentration of the DNA (1 O.D. at 260 nm equals 50 µg ml^{-1}dsDNA).

Detection of thymine dimers

The blot papers (GB002, Schleicher & Schuell, Dassel, Germany) and the nylon membrane (Nytran N2, Schleicher & Schuell) were soaked in a solution containing 3 M NaCl and 0.3 M Na-citrate and placed on a slot or dot blot manifold (Minifold I, Schleicher & Schuell). DNA samples were transferred to the membrane and washed once with TE buffer. The membrane was dried for 1 h at 80°C to immobilize the DNA. Subsequently, the membrane was incubated for 1 h in PBS-T [phosphate buffer saline: 0.14 M NaCl, 3.4 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4 + 0.1 % (v/v) Tween 20] with 5 % (w/v) skimmed milk powder to block the non-specific sites. Thereafter, the membrane was incubated with the primary antibody (anti-thymine dimer KTM53, Kamiya Biomedicals, Seattle, USA; diluted 1:10000 in PBS-T) for 2 h at room temperature and then washed (3 x 10 min) with PBS-T. Afterwards the membrane was incubated with the secondary antibody [anti-mouse IgG (Fab specific) peroxidase conjugate, Sigma, Saint Louis, Missouri, USA; diluted 1:10000 in PBS-T with 5 % skimmed milk powder] for 1 h at room temperature and washed with PBS-T (4 x 10 min). Finally, the membrane was placed in a detection reagent (Renaissance, NEN Life Science Products, Cologne, Germany) for 1 min before scanning (Kodak Digital Science, Image Station 440 CF, New Haven, CT, USA). The results were evaluated by using the software provided by the manufacturer.

Calibration standard for thymine dimer quantification

In order to establish a standard with known thymine dimer frequency the plasmid pBSK (obtained from R. Marschalek, Frankfurt) was used. For isolation of RNA-free plasmid DNA the DNA Midiprep I Kit from peQLab (Erlangen, Germany) was used. The plasmid has a size of 2961 bp which corresponds to 3.25 x 10^{-12} µg. In the genome 326 thymine pairs occur. Thus 1 µg of the plasmid contains 1.1 x 10^{14} thymine dimers when all possible pairs are dimerized. In order to almost completely induce all thymine dimers the plasmid was irradiated with the transilluminator, as described earlier, for 60 min. Various concentrations (1-1000 ng) of irradiated pBSK was loaded on dot blots. The frequency F of thymine dimers (T^T) per megabase pair is calculated with the following equation, where T^T is the number of thymine dimers in the irradiated plasmid DNA and M the total DNA mass:

$$F = T\hat{}T / (9.11 \times 10^{14} * M) * 10^{6}$$

Stability of thymine dimers at 4 and -20°C

The plasmid pBSK was irradiated with the transilluminator as described above and the sample was divided into two equal parts. One part was stored overnight at 4°C in a refrigerator and the second part at -20°C in a freezer. Next morning, the DNA was blotted to test the stability of thymine dimers as well as the overall concentration of the DNA to evaluate the effects of the two temperatures.

RESULTS

We first isolated the plasmid DNA (pBSK) from *E. coli* and determined the yield. Thereafter, the DNA from control and UV-irradiated samples of various cyanobacteria, phytoplankton and macroalgae were isolated, and the total yield was determined. Purified plasmid DNA and the DNA from studied organisms had their absorption maximum at 260 nm (data not shown).

Formation of thymine dimers in plasmid DNA and test organisms after UV irradiation was determined by blotting and chemiluminescence method. Various concentrations (1-1000 ng) of UV irradiated plasmid DNA (for the determination of a calibration curve) and equal amounts of the DNA from test organisms were loaded onto the nylon membrane. Figure 1 represents the blotting pattern of both plasmid DNA (lanes A; 1-8) and DNA from the cyanobacterium *Nostoc* sp. (lanes B; 1-5) after increasing times of UV radiation. There was a gradual increase in the intensity of the luminescence with increasing concentrations of plasmid DNA (Fig. 1; lanes A, 1-8). Thymine dimers were detectable with this method at DNA concentrations as low as 1 ng. Similarly, there was a gradual increase in the intensity of the luminescence with increasing UV irradiation times in *Nostoc* sp. (Fig. 1, lanes B, 1-5).

The formation of thymine dimers (T^T/Mb) in plasmid DNA was determined and a calibration curve was plotted (Fig. 2). Similarly, the frequency of thymine dimers formed after different durations of UV radiation was calculated in *Nostoc* sp. (Fig. 3). The quantitative method for the determination of thymine dimers works equally well for the phytoplankton and the macroalgae; results of which have been shown in Table 1 together with other cyanobacteria. Figure 3 and Table 1 show an induction in the frequency of thymine dimers with increasing UV irradiation time. It is pertinent to mention that the UV-irradiated DNA samples should not be frozen since this results in a loss of thymine dimers (Fig. 4). Although the total DNA concentration was not affected by the cold treatment the quantification of the dots (Fig. 4 insets) shows a loss of about 20-25 % thymine dimers in the UV irradiated DNA samples placed overnight at -20°C (Fig. 4). This loss could be due to structural changes of the thymine dimers by freezing.

DISCUSSION

The survival of organisms depends on the accurate transmission of the genetic information from one cell to its daughters. Such faithful transmission requires not only extreme accuracy in replication of DNA and precision in chromosome distribution, but also in the ability to survive spontaneous and induced DNA damage while minimizing the number of heritable mutations (Zhou and Elledge 2000). UV-induced DNA damage and its photoenzymatic and nucleotide excision repair have been known from diverse organisms (Mitchell and Karentz 1993, Britt 1996, Pakker *et al.* 2000, Zhou and Elledge 2000).

This paper presents a simple and efficient method for the quantitative analysis of thymine dimers in various aquatic primary producers such as cyanobacteria, phytoplankton and macroalgae, in order to better understand the role of UV radiation in eliciting mutagenic effects. A number of methods for DNA isolation from diverse organisms are in practice (Karentz *et al.* 1991, Rogers and Bendich 1994, Rudi *et al.* 1998, Fiore *et al.* 2000, Pakker *et al.* 2000, Perdiz *et al.* 2000, Tillett and Neilan 2000). Our protocol for DNA isolation is simple, rapid and inexpensive, providing high quality DNA from a wide range of organisms. A number of workers use NaI which is not only expensive and environmentally hazardous but degrades in a short period of time (Fiore *et al.* 2000). NaCl was used instead which is not only cheaper but also has the advantage of reducing the amount of RNA and hence making treatment with RNase unnecessary (Fiore *et al.* 2000). Similarly, we do not use phenol, which creates environmental hazards.

A number of methods are in use to determine the DNA damage in a variety of organisms (O'Brine and Houghton 1982, Freeman *et al.* 1986, Mitchell *et al.* 1991, Van Loon *et al.* 1992, Buma *et al.* 1995, Hidema *et al.* 1999, Douki *et al.* 2000, Pakker *et al.* 2000, Perdiz *et al.* 2000, Sommaruga and Buma 2000). UV-induced DNA degradation has been reported in the cyanobacterium *Synechocystis* by using radioactive methods (O'Brine and Houghton 1982) and showing percentage radioactivity lost from DNA as a measure for DNA degradation. An alkaline agarose gel method for quantifying single strand breaks in nanogram quanti-

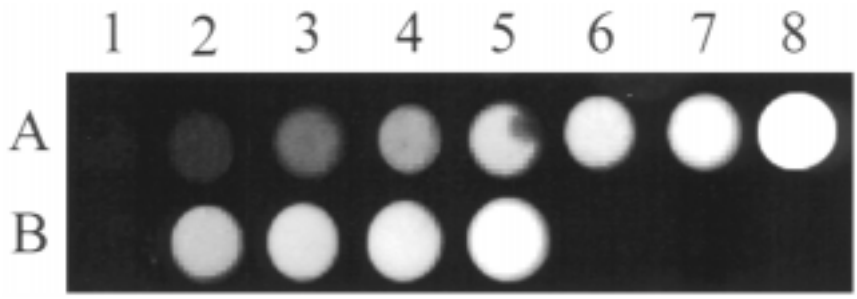

Fig. 1. Dot blot of both plasmid DNA (pBSK) and DNA form *Nostoc* sp. Lanes A (1 - 8), plasmid DNA (1, 2, 5, 10, 20, 50, 100 and 1000 ng, respectively). Lanes B (1-5), DNA from *Nostoc* sp. after different durations of UV radiation (0, 15, 30, 60 and 120 min of UV radiation, respectively)

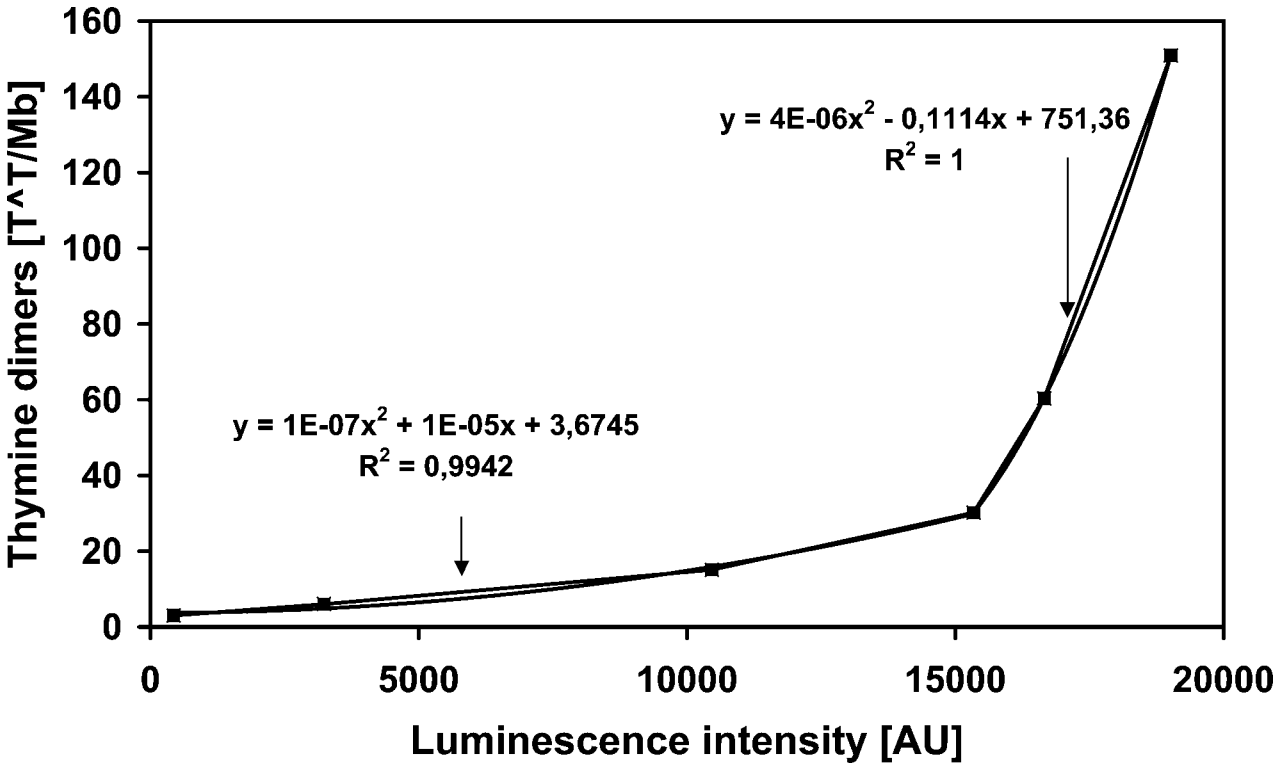

Fig. 2. Calibration curve plotted from blots of plasmid DNA (1-50 ng) as shown in Fig. 1. For details see text

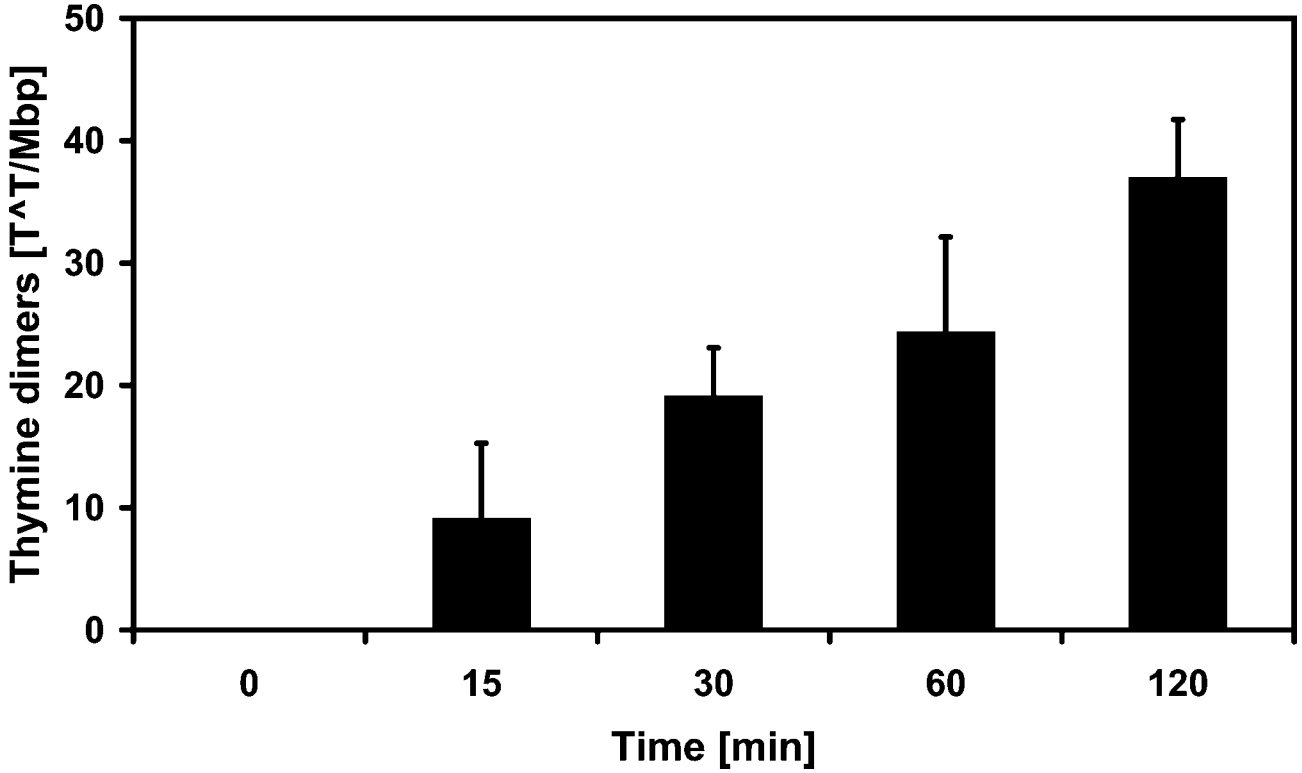

Fig. 3. Frequency of thymine dimers in *Nostoc* sp. after UV-B irradiation for different durations. For details see text

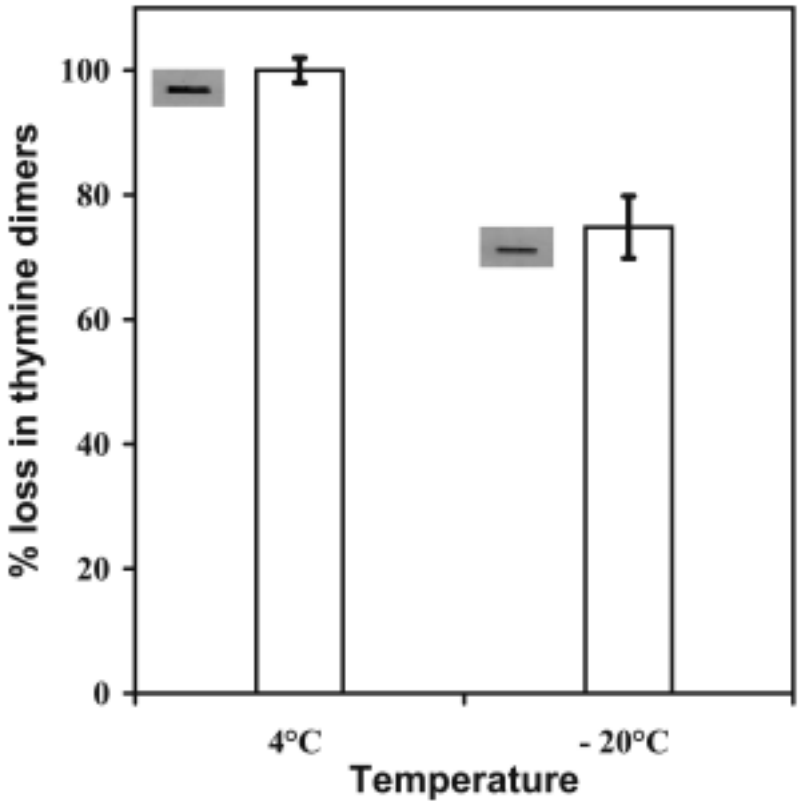

Fig. 4. Loss in thymine dimers after overnight freezing at -20°C of UV irradiated plasmid DNA (pBSK). The slot blots (insets) are the representative of ten different but identical experiments on the basis of which percentage loss in thymine dimer was calculated

Table 1. Frequency of thymine dimers in various organisms after UV (10 W/m^2) irradiation for different durations

Organism	T^T/Mbp				
	UV irradiation [min]				
	0	15	30	60	120
Cyanobacteria					
Anabaena sp.	0	8.23 ± 4.30	16. 45 ± 5.22	22.42 ± 6.52	39.65 ± 6.65
Scytonema sp.	0	6.52 ± 4.21	13.62 ± 4.42	18.68 ± 5.95	35.55 ± 5.22
Phytoplankton					
Euglena gracilis	0	7.52 ± 4.63	15.47 ± 4.21	21.82 ± 7.42	36.25 ± 4.45
Gyrodinium dorsum	0	6.82 ± 3.67	14.58 ± 4.45	19.98 ± 6.45	32.55 ± 5.45
Macroalgae					
Porphyra umbilicalis	0	5.56 ± 2.33	9.98 ± 2.50	14.56 ± 2.68	26.87 ± 3.58
Ceramium rubrum	0	5.22 ± 2.15	8.68 ± 2.11	15.58 ± 3.55	28.57 ± 3.69

ties of nonradioactive DNA was developed by Freeman *et al.* (1986). Another method for cyclobutane dimer detection was presented by Mitchell *et al.* (1991). They first labeled the DNA by radioactive substances followed by agarose gel electrophoresis and densitometric analysis and finally digesting with endo III and endo V before analyzing on sequencing gels. An immunochemical assay was improved by Van Loon *et al.* (1992) for quantitative detection of DNA damage. This technique was based on the enhancement of the radiation-induced single-strandedness which was determined by using a monoclonal antibody. Buma *et al.* (1995) developed an immunofluorescent thymine dimer detection method by labeling dimers with antibody followed by a secondary antibody (fluorescein isothiocyanate) staining and finally visualization of DNA damage with flow cytometry or fluorescence microscopy. More or less the same method was used in subsequent publications by Pakker *et al.* (2000) and Sommaruga and Buma (2000). Yet another method for measurement of thymine photoproducts by using an electrospray-mass spectrometer was presented by Douki *et al.* (2000). Most of the methods discussed above present relative data on DNA damage. We present a simple and efficient quantitative method to determine the frequency of thymine dimers in a variety of organisms in relatively short period of time by using blotting and chemiluminescence methods. This method neither requires radioactive labeling of DNA nor its detection by agarose gel electrophoresis (where ethidium bromide is being used to stain the DNA) and thereby eliminating the possibilities of health hazards. This method permits the measurement of low as well as high levels of DNA lesions in nanogram quantities of DNA and can be used for the cultured as well as naturally occurring organisms. Once the frequency of the thymine dimers is determined, it could well be correlated with the survival of the organisms.

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