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Full Length Research Paper

Development of a DNA-dosimeter system as biomarker to monitor the effects of pulsed ultraviolet radiation

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To report the effects of pulsed ultraviolet (PUV) radiation, we have developed a reliable biological monitoring system based on two approaches. Firstly, a conventional method was used to measure the number of colonies by the estimation of viable and cultivable bacteria before, and after each exposure to PUV radiation. The second method was a DNA-dosimeter system based on polymerase chain reaction (PCR) 16S ribosomal DNA (rDNA) and on terminal restriction fragment length polymorphism (T-RFLP) analysis. PCR was performed using 27F and 905R primers to replicate a fragment of the rDNA gene. The comparison of inactivation kinetic results obtained by a classic account of viable and cultivable bacteria (UV dose/ response) and the analysis of DNA-dosimeter determined by PCR amplification and peak-profiles T-RFLP; shows a correlation between the reduction of the colony-forming ability of *Pseudomonas aeruginosa* and the progressive decrease of 16S rDNA PCR products and of relative peak area of a specific terminal restriction fragment (T-RF).

Key words: Pulsed UV light, *Pseudomonas aeruginosa*, viable but non-culturable (VBNC) bacteria, 16S rDNA, terminal restriction fragment length polymorphism (T-RFLP).

INTRODUCTION

The light generated by pulsed ultraviolet (PUV) lamps consists of a continuous broadband spectrum from deep UV to the infrared (IR), especially rich in UV range below 400 nm, which is germicidal. In PUV light system, UV-light is pulsed several times per second and each pulse lasts between 100 ns and 2 ms (Sharifi-Yazdi and Darghahi, 2006).

PUV light is a non-thermal, high-peak power technology that consists of intense flashes of broad-spectrum white light with wavelengths from 200 nm in the UV to 1000 nm in the near-IR region (Rowan et al., 1999).

Each pulse may have up to 90000 times the intensity of sunlight at sea level, and may last only a few hundred millionths of a second, and thus a PUV light system can produce very high peak power pulsed light in a very short time. Because of its high peak power, PUV light has been successfully used as a sterilization tool to kill bacteria and fungi in foods (Bialka et al., 2008) and water (Sharifi-Yazdi and Darghahi, 2006). The killing effect is 4 to 6 times higher than that of the conventional continuous UV light at the same energy level (MacGregor et al., 1998).

The wavelength for UV processing ranges from 200 to 280 nm, called the germicidal range, since it effectively inactivates the microorganisms. The effectiveness of germicidal UV light in biological inactivation arises primarily from the fact that DNA molecules absorb UV photons between 200 and 300 nm, with peak absorption at 254 nm (Ben Said et al., 2011). This absorption creates damage in the DNA by altering nucleotide base pairing; thereby creating atypical linkages between adjacent nucleotides on the same DNA strand. This

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damage occurs particularly between pyrimidine bases that result in an inhibition of replication and, in case of lethal doses, in a loss of reproducibility. However, microbes possess several mechanisms to enable cell survival following UV exposure.

Two well known types of mutagenic lesions in UV irradiated DNA were determined; Cyclobutane Pyrimidine Dimers (CPDs) formed between the C-4 and C-5 positions of adjacent thymidine or cytosine residues, and pyrimidine (6–4). Pyrimidine (6–4) photoproducts formed between the C_6 and C_4 position of adjacent pyrimidine residues, most often between T-C and C-C residues (Douki et al., 2003).

However, UV disinfection is noted to have some problems, one of them is reactivation. In fact, to a certain extent, DNA damage can be tolerated by the cell until repair occurs (Zimmer and Slawson, 2002). The mechanism by which, microorganism recovers replication activity; through a direct reversion of thymine dimers is called photoreactivation (Douki et al., 2003). This process is catalysed by the DNA repair enzyme photolyase and requires visible light. Apart from photoreactivation, numerous light-independent repair mechanisms exist that are regulated by the expression of the single-strand DNA binding protein RecA (Makarova et al., 2000).

The aim of this study was to monitor the effectiveness of PUV light to inactivate tested bacteria using two bio-dosimetry approaches: (i) study of bacterial response to an increasing number of PUV irradiation (dose/response); (ii) use of PCR assay amplified a 16S rDNA fragment and T-RFLP analysis of PCR products and (iii) compare between results obtained by classic and molecular bio-dosimetry techniques.

MATERIALS AND METHODS

Bacterial strains

Pseudomonas aeruginosa used in this study was obtained from American Type Culture Collection (ATCC 15442). Cultures were grown in Luria-Bertani broth (LB) [10 g tryptone; 5 g yeast extract; 10 g NaCl] or LB agar (LBA) (10 g tryptone; 5 g yeast extract; 10 g NaCl 15 g/L agar). Saline [0.85% (wt/vol) NaCl] was used for cells suspensions during UV irradiation.

PUV radiation

The PUV system is developed by the combination with power and flash UV lamp technology. PUV light was differed from the traditional continuous UV light by the much higher irradiance of UV illumination and reduction of exposure time. Indeed flash lamps commonly operate with pulse lengths ranging from a few tens of milliseconds to over milliseconds.

UV irradiation for polychromatic UV source (UV pulse lamp) was measured using a potasium iodide/iodate actinometry (KI/KIO_3) according to Rahn et al. (2003).For this study, UV dose determined by chemical actinometry was equal to 5.72 mJ/cm^2 per UV-pulse.

In order to reduce the photo-thermal effect of PUV light due to visible light and IR, the PUV system was equipped with a ventilator.

UV-irradiated bacteria

For dose/response relationship and reactivation experiments, the strain of *P. aeruginosa* was cultured in Luria-Bertani broth (LB). Bacterial suspension was diluted in saline Phosphate Buffer (PBS) in order to obtain a concentration ranged from 1 x 10^5 to 1 x 10^6 cfu/ ml. Then, the bacterial suspensions were used for irradiation experiments. A volume of 20 ml of the prepared suspensions was transferred into a standard Petri dish for the eventual exposure to an increasing number of PUV-light.

Viable cell counts

Viable cell counts were taken before and immediately after UV exposure. A 100 µl portion of each irradiated samples was removed in order to prepare serial dilutions in PBS buffer. A volume equal to 100 µl of the appropriate serial dilutions was spread in duplicate onto LB agar. The number of colony-forming unit (CFU/ml) or a number of viable and cultivable bacteria was determined after 24 h of incubation at 37°C. The fraction of viable and cultivable bacteria was calculated by dividing the number of CFU in the UV-treated sample (N) by the number of CFU determined at time zero before UV irradiation (N_0).

DNA extraction from *P. aeruginosa*

The genomic DNA of *P. aeruginosa* was extracted immediately before and after irradiation by different doses of UV-C light and after rest times conditions the DNA extraction using DNA extraction kit UltraClean_Soil DNA™ Isolation Kit (Mo Bio Laboratories, Int., Carlsbad, CA) following the manufacturer's instructions. The quantity and quality of the DNA were checked by agarose gel electrophoresis (1%, w/v) in TAE buffer. The image of the stained gel was photographed (Gel Doc 1000; Bio Rad) and analysed (Molecular Analyst software; BioRad).

PCR conditions

For 16S rDNA amplification, the universal bacterial primer set with 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 905R (5'-CCGTCAATTCATTTGAG-3') primers was used (Kasuga et al., 2007). The 5' end of forward primer (27F) was labeled with a 6-carboxylfluorescein-derived phosphoramidite fluorochrome (6-FAM). PCR amplification was conducted in triplicate by using an AmpliTaq Gold DNA polymerase kit (Applied Biosystems, Foster City,CA). The termal cycling conditions consisted of initial heat denaturation at 95°C for 10 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min. A final extension was then performed at 72°C for 10 min. The amplified rDNA were quantified using a NanoDop® ND-1000 spectrophotometer (NanoDop Technologies, Wilmington, DE).

T-RFLP analysis

The triplicate PCR products for each irradiated samples were mixed and purified using a MinElute PCR Purification kit (QIAGE, Hilden, Germany). The DNA concentration was quantified using a NanoDop® ND-1000 spectrophotometer (NanoDop Technologies, Wilmington, DE).

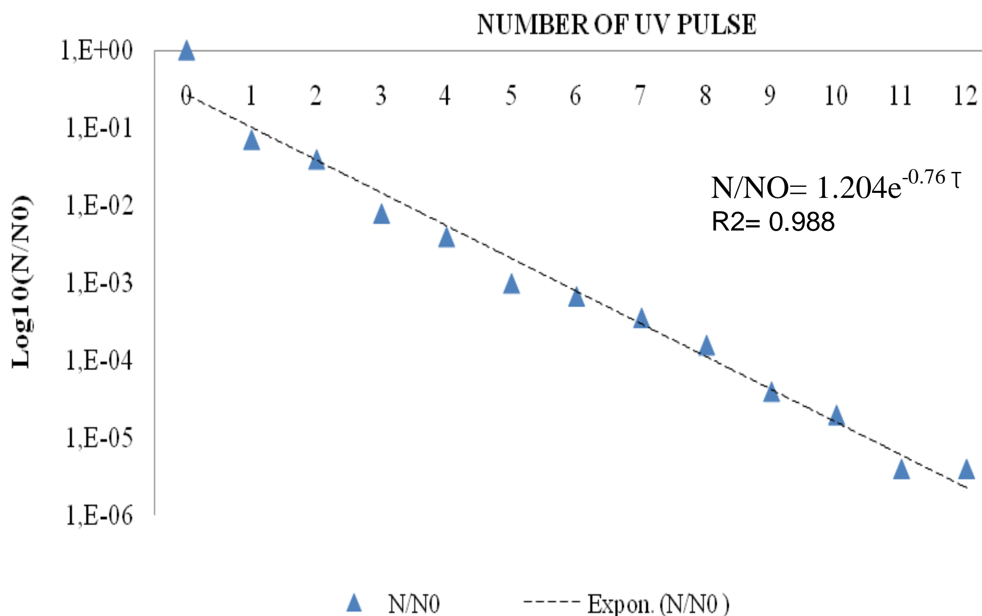

Figure 1. The Kinetic of *P. aeruginosa* ATCC 15442 inactivation following exposure to UV-C radiation according to the model of Chick-Watson where; *y*: Reduction = N/N_0 with N_0: Number of viable cell before exposure to UV light, *N*: Number of viable cell after exposure to UV-C radiation; x = $I^n T$ with *I*: UV intensity (mW/cm^2), *T*: Number of PUV light; $n = 1$. Where error bar are not shown, differences between duplicates were not detected.

Restriction enzyme digestion was conducted in triplicate. The PCR products were digested with 10 U of the tetrameric restriction enzyme *Hha*I (TaKaRa BIO Inc., Otsu, Japan) in a 20 µl volume according to the manufacturer's instruction. The digested products were purified using a QIAquick Nucleotide Removal Kit (QIAGEN). The 6-FAM-labeled fragments were analysed with an ABI Prism® 310 Genetic Analyser (Applied Biosystems). Fragment analysis was carried out by using GeneMapper™ v3.0 software (Applied Biosystems). The detection threshold for terminal-restriction fragments (T-RFs) was set to 100 relative fluorescent units (RFU) for the software. Relative abundance of T-RFs was calculated based on their peak area.

RESULTS

The inactivation kinetic of *P. aeruginosa*

The inactivation rate of *P. aeruginosa* was function of UV-C dose. The germicidal dose was expressed as the product of UV radiation intensity (*I*) and number of PUV light (*T*) (Figure 1).

The lethal effects of pulsed light can be attributed to its rich broad-spectrum UV content, its short duration, and high peak power, which play a major role in bacterial inactivation (Sharifi-Yazdi and Darghahi, 2006). Indeed the UV region is crucial to the efficiency of PUV light treatment. It has been confirmed that no killing effect is achieved if a filter is included to remove the UV wavelength region below 320 nm (Takeshita et al., 2003).

UV dose-response

In order to study the behavior or the response of tested bacteria to an increasing UV dose (dose/response), the mathematical model of Chick-Watson was used according to Hassen et al. (2000):

$$N/N_0 = A \exp(-kI^n T) \quad (1)$$

Where, N_0: Number of viable cultivable bacteria before exposure to UV light; *N*: Number of viable cultivable bacteria after exposure to PUV light; *A*: constant corresponding to bacteria retaining viability following UV irradiation; *K*: Coefficient of lethality; *I*: The UV-C intensity expressed in mW/cm^2; *T*: number of UV pulse and *n*: Threshold level of series-event mode; $n = 1$ for the first order Chick-Watson model. The constants *K* and *A* were determined by linear regression.

The inactivation kinetic (dose/response) according to the model of Chick-Watson (Equation 1) shows that the irradiation of *P. aeruginosa* by 8 UV pulses is sufficient for 99.99% inactivation of colony-forming ability, which corresponds to a UV dose equal to 45.76 mJ.cm^{-2}. This UV dose is nearest of the UV fluency generally used in Europe and the USA for the disinfection of drinking water. Indeed, according to the literature, 40 mJ.cm^{-2} is enough to inactivate 4 Unit-log$_{10}$ of pathogenic bacteria as *Legionella*, enteric viruses, *Cryptosporidium* oocysts and

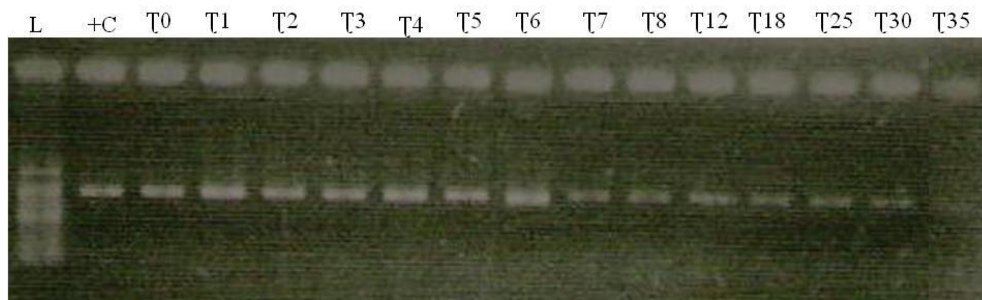

Figure 2. Agarose gel electrophoresis of PCR products generated from irradiated *P. aeruginosa* with the primer set 27F and 905R. Image of a 1% agarose gel stained with ethidium bromide. With, L: 100bp ladder; +C: positive control, T_n: Number of PUV light.

Giardia cysts (US-EPA, 2003).

By the analysis of irradiated *P. aeruginosa* kinetic curve, we can conclude that 8 UV pulses were sufficient to inactivate 99.99% of viable and cultivable bacteria according to a conventional applied dose.

At this stage of research, the question is the equivalent UV dose equal to 8 UV pulses effective or not for inactivating bacteria at molecular level?

To answer this question, and to predict biologically effective of applied UV doses, DNA dosimeter system based on 16S rDNA PCR amplification and T-RFLP analysis were used to monitor effects of PUV radiation.

PUV light DNA dosimeter

Based on UV-inactivation’s kinetic curve of *P. aeruginosa*, the tested bacteria were exposed to 8, 12, and 18 UV pulses. Applying these doses resulted in the inactivation of 99.99% inactivation of bacteria, where the loss of cultivability of tested bacteria was with or without subsequent reactivation. Moreover, the bacteria to a higher number of UV pulses (25, 30, and 35 UV pulses) were exposed in order to explore the effects of PUV irradiation on bacterial DNA at a sub-lethal doses.

DNA dosimeter analyzed by polymerase chain reactions (PCR)

The study of DNA-dosimeter was obtained by the analysis of 16S rDNA PCR products for the same tested bacteria and for different irradiation conditions using 27F and 905R primers. The amplified fragments were to be approximately 1500 base pairs long (Figure 2).

PCR inhibition was detected already by agarose gel electrophoresis prior to T-RFLP analysis to check the size of the PCR products (Figure 2). An intense band was visible for the unirradiated sample and irradiated samples with a low PUV. The signal strength of the band was reduced directly after irradiation (8 UV-pulses).

During PCR amplification, primers and *Taq polymerase* across different obstacles (photoproducts) conducts continual disruption of PCR amplification in function of an increase number of PUV light.

The comparison between UV dose /response and DNA dosimeter

The comparison of inactivation kinetic obtained by a classic account of viable and cultivable bacteria (Figure 1) and the analysis of DNA-dosimeter determined by PCR amplification (Figure 2) shows in part, the relationship between the progressive decrease of PCR products and the reduction of the colony-forming ability of *P. aeruginosa*.

The exploitation of DNA-dosimeter determined by 16S rDNA PCR of *P. aeruginosa* was obtained by the analysis of PCR products using Molecular Analyst software (BioRad), by which a fluorescence intensity area (FIA) of stained DNA bands was determined, with ethidium bromide (Figure 3).

According to the first used bio-dosimetry system (dose/response), 8 UV pulses were sufficient to inactivate 99.99% of viable and cultivable bacteria. This number of UV pulses can allow the inhibition of nearly 35% of 16S rDNA amplification *in vitro* by PCR using 27F and 905R primers and *Taq polymerase* for DNA extension (Figure 3). Despite the partially inhibition of PCR amplification, nearly 65% of amplified 16S rDNA can be ensured *in vitro*. This percentage revel that, the equivalent dose of 8 UV pulses allows the inhibition of 99.99% of bacterial cultivability in usual media, but not the DNA replication, and thereby, bacterial viability and toxicity.

We can conclude that, the information obtained restrictively from the simple count of viable and cultivable bacteria is incomplete. Indeed, some bacteria lose the cultivability on appropriate growth media but can exhibit signs of metabolic activity and thus viability (Armisen and

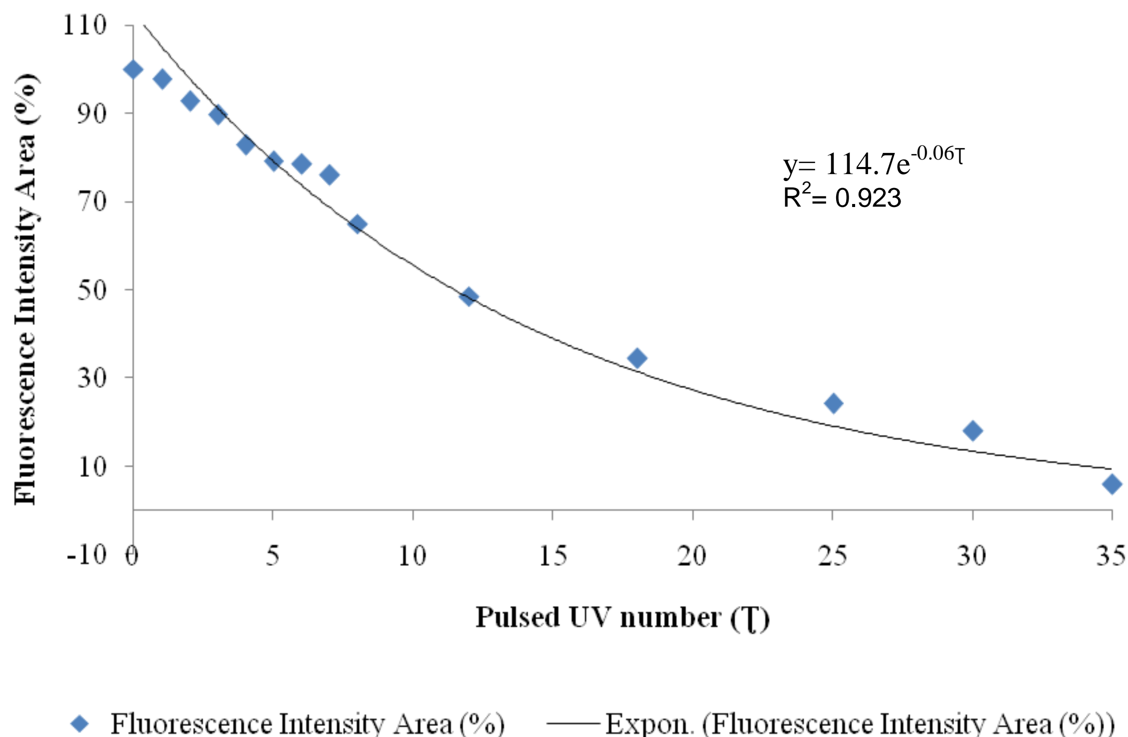

Figure 3. DNA-dosimeter determined by fluorescence intensity area (%) of PCR products of *P. aeruginosa* using 27F and 905R primers.

Servais, 2004). The presence of these viable but non-culturable (VBNC) bacteria in natural environment could be important from a sanitary point of view as some authors (Ben Said et al., 2010; Servais et al., 2009; Pommepuy et al., 1996) suggested that pathogenic VBNC bacteria could maintain their virulence being a potential reservoir of disease.

In addition, after irradiation by 12 UV pulses (≅ 68 $mJ.cm^{-2}$), nearly 48% of 16S rDNA could be amplified (Figure 3). This percentage shows the ability of viable but non cultivable bacteria not yet reactivated to ensure DNA replication and bacteria resuscitation. However, when the number of UV pulses were increased over 12 pulses, a significant inhibition of PCR amplification was shown for consequent accumulation of photoproducts generated by germicidal wavelengths of PUV light. In fact, UV-induced DNA lesions such as CPDs show differential effects on DNA conformation, impairing their regulatory functions and other dynamic processes. Their UV-DNA effects have a repercussion on DNA replication *in vitro* using PCR. Thus, an increasing number of PUV light can cause mutations in the primer binding sites on the template strand or a blockage of extension step assumed by *Taq polymerase*. Noted that, the inhibition of rDNA amplification for post-irradiated strain *in vitro* is similar to what is going *in vivo* at bacterial DNA level during replication and transcription.

PUV light DNA dosimeter analyzed by "peak-profiles T-RFLP"

A semi-quantitative molecular technique was developed for rapid analysis of PUV light effects on rDNA amplification. The technique employed PCR in which one of the two primers used was fluorescently labeled at the 5' end, and was used for genes encoding 16S rDNA from total community DNA of unirradiated and irradiated *P. aeruginosa*. The PCR product was digested with restriction enzymes, and the fluorescently labeled terminal restriction fragment was precisely measured by using an automated DNA sequencer (Kasuga et al., 2007).

Figure 4 shows the electropherograms of 16 rDNA T-RFLP profiles before and after each irradiation by PUV light.

The analysis of terminal restriction fragment length polymorphisms

Computer-simulated analysis of terminal restriction fragment length polymorphisms (T-RFLP) for UV-post irradiated *P. aeruginosa* sequences showed that with proper selection of PCR primers (27F and 905R) and restriction enzyme (*Hha*I) (Figure 4), there is no

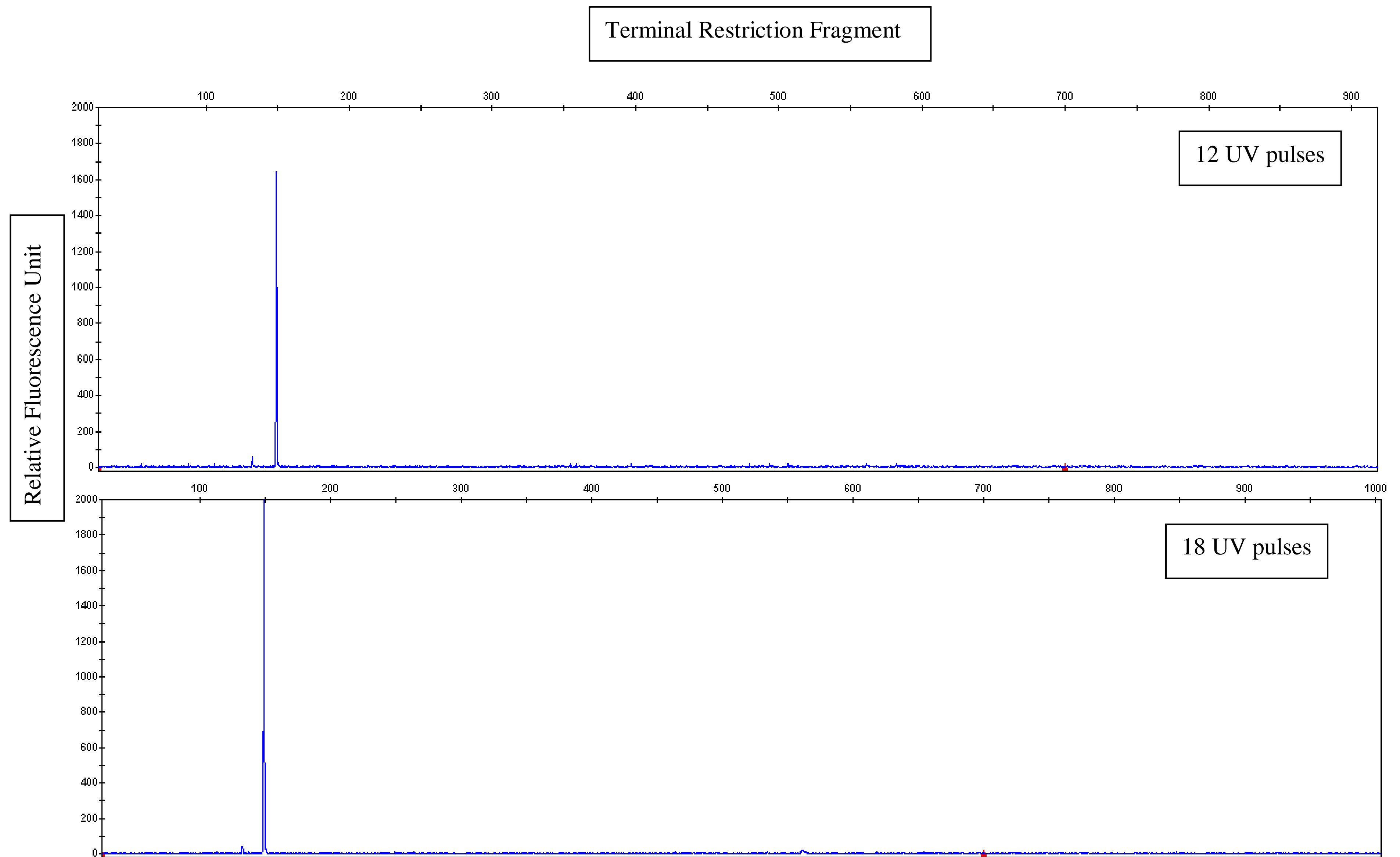

Figure 4. Electropherograms of T-RFLPs of *Hha*I digested 16S rDNA amplified from unirradiated and irradiated *P. aeruginosa* ATT15422 by an increasing number of PUV light.

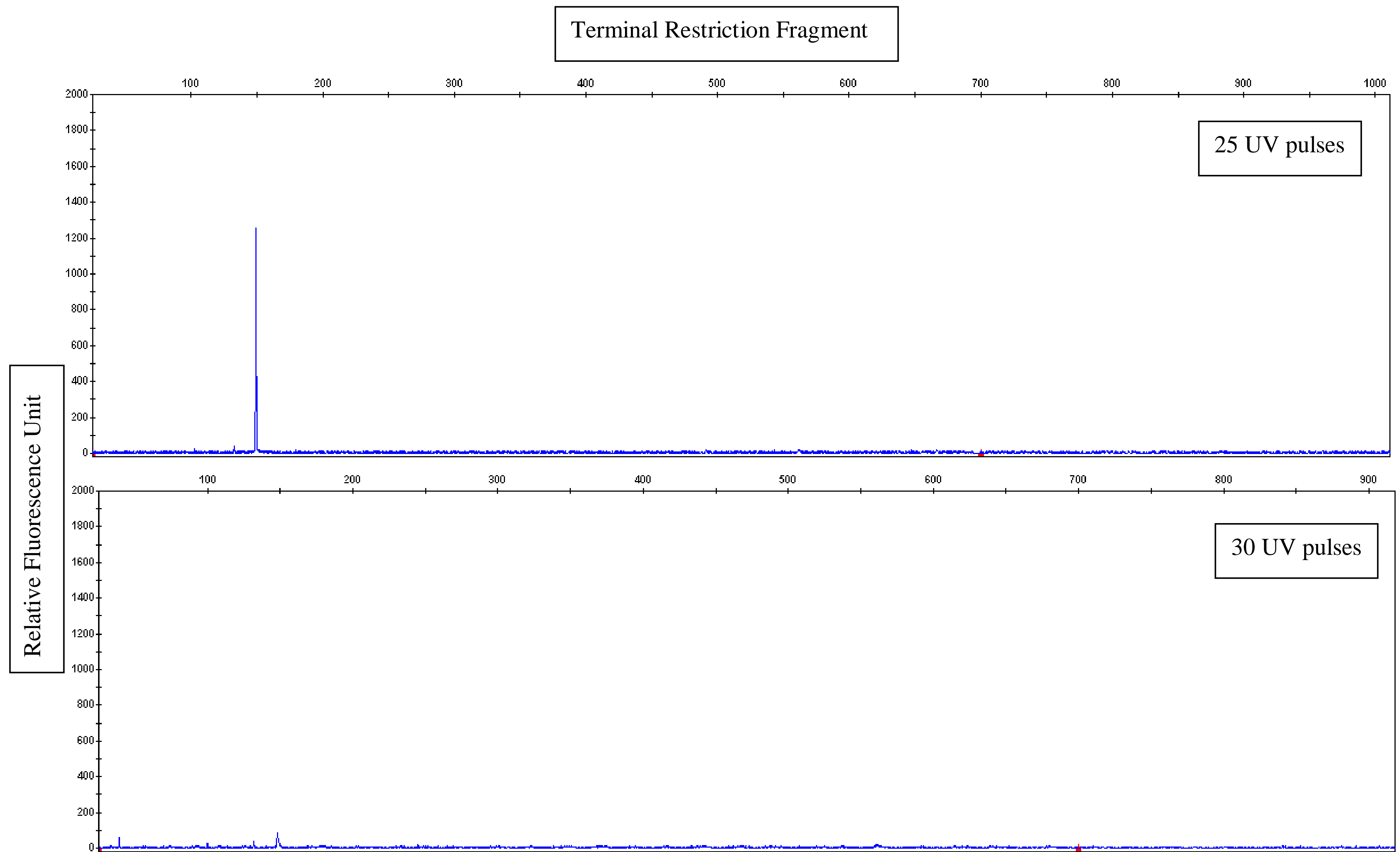

Figure 4. Contd.

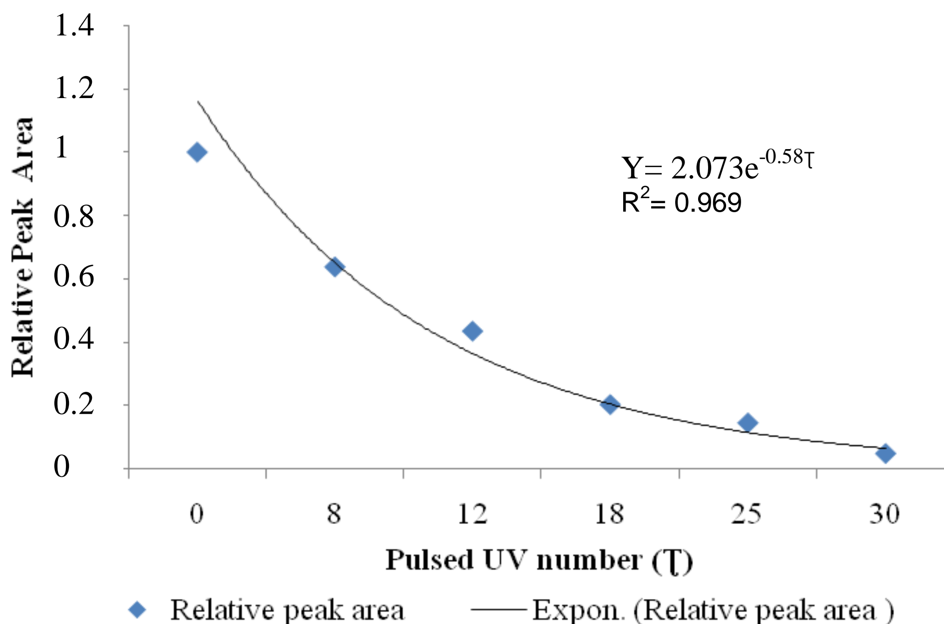

Figure 5. Relative peak area of T-RFs of irradiated *P. aeruginosa* ATT15422 by an increasing number of PUV light.

difference in terminal restriction fragment (T-RF) sizes; Indeed, all profiles consisted of identical and single T-RF nearly 148 pb (± 1pb); however, some of T-RF had different peak area. Relative peak area (RPA) was calculated in percentage by dividing peak signal determined for the irradiated bacteria by the total signal determined for the control test before UV irradiation (Urakawa et al., 2000). The measure of relative peak area or relative peak height was presented in Figure 4. The difference in "peak-profiles T-RFLP" was probably due to the interruption of PCR steps. This partial or complete interruption of PCR amplification was directlyrelated to the applied number of PUV light (Figure 5).

We can model the results of DNA-dosimeter determined by T-RFLP analysis according to Chick-Watson model with modification:

$$RPA_{Ţ} / RPA_{Ţ0} = A_{CPD} \exp(-k_i Ţ) \quad (2)$$

With, $RPA_{Ţ0}$: RPA calculated at time zero before UV irradiation; $RPA_{Ţ}$: RPA calculated after irradiation by a number ($Ţ$) of PUV light; k_i: inhibition coefficient of a specific terminal restriction fragment (T-RF); and A_{CPD}: photoproduct accumulation rate.

T-RFLP technique was based on the determination of relative peak area of terminal restriction fragments (T-RFs) generated by a restriction enzyme after PCR step. For consequence, by T-RFLP analysis we can "zoom" the effects of PUV light on bacterial DNA.

In addition, after irradiation by PUV light, there was a decrease of a relative peak area ($RPA_{Ţ}$) of the specific T-RF for irradiated DNA (Figures 4 and 5). For instance, after irradiation by 8 pulses UV light and inactivation of 99.99% of viable and cultivable bacteria; the relative peak area of T-RF is equal to 64% compared to the relative peak area determined for *P. aeruginosa* at time zero before UV irradiation.

Moreover, after 12 UV pulses; the $RPA_{Ţ}$ (%) is equal to 43% of the single T-RF comparing to $RPA_{Ţ0}$ at time zero before UV irradiation. According to the inactivation kinetic of tested bacteria (Figure 1), this applied dose allowed the loss of bacteria cultivability in usual media with subsequent reactivation.

Also, the persistence of a specific T-RF despite the increasing irradiation by a PUV light shows a higher intrinsic resistance of studied *P. aeruginosa* against UV irradiation. The disappearance of T-RF was shown after 30 UV pulses (Figure 4).

The relatively abundance of bacteria in irradiated samples given by DNA-dosimetry results, strengthens the existence of different "bacterial viability form" among the same irradiated bacteria. Indeed, the single T-RF can include viable but non cultivable (VBNC) bacteria not yet reactivated, active but non cultivable (ABNC) bacteria and, VBNC-UV inactivated bacteria. This fact was not taken into consideration in the classical evaluation method.

Accordingly, the application of DNA-dosimetry to estimate the effectiveness of UV disinfection and the relative abundance of bacteria before and after treatment of water was shown to be useful.

Conclusion

The public health risk is thus not only a function of the abundance of the microorganism's contaminants in water, but also of their capacity to survive in the receiving environments to maintain their virulence (Ben et al., 2010). It would be pertinent to take into consideration at the same time the effectiveness of the disinfection system process and to develop sensible techniques such as molecular methods in order to compare survival of the bacteria upstream and downstream the disinfection system and to study the infectivity and the virulence of the microorganisms treated by UV light (continuous UV radiation or PUV light).

In addition, the DNA- dosimeter based approach presented is a promising tool for biological risk assessment during UV-based technical processes. It directly records the response of bacteria to UV radiation independently of cultivability in usual media.

The DNA-dosimetry methods should be standardized to provide accurate estimation of water quality instead of bio-dosimetry which is based only on the determination of viable and cultivable bacteria after UV treatment.

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