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ORIGINAL ARTICLE

Development of a DNA-dosimeter system for monitoring the effects of pulsed ultraviolet radiation

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Abstract To improve the ultraviolet (UV) water disinfection process and to better understand the impact of the harmful effects of germicidal radiation on the DNA molecule, we have developed a reliable biological monitoring system based on PCR 16S ribosomal DNA (rDNA) and terminal restriction fragment length polymorphism analysis. The PCR analysis was performed using the bacteria-specific 27F and 905R primers to replicate a fragment of the rDNA gene. This new and versatile method can be used to evaluate the effects of direct UV radiation on DNA (UV dose/response) and to estimate the potential of bacteria to mitigate UV lethal effects via photoreactivation and dark repair.

Keywords Pulsed UV light · *P. aeruginosa* · VBNC bacteria · Reactivation · 16S rDNA · T-RFLP

Introduction

Ultraviolet (UV) light is a proven means for the disinfection and remediation of water. In many cases, it may be preferable over more traditional chemical disinfectants because of the tendency of such methods to produce disinfection by-products of regulatory concern.

Pulsed UV (PUV) light is a nonthermal, high-peak power technology that consists of intense flashes of broad-spectrum white light with wavelengths ranging from 200 nm in the UV to 1,000 nm in the near-infrared region (Rowan et al. 1999). The intensity of each pulse may be up to 90,000-fold greater than that of sunlight at sea level, and each pulse may last only a few hundred millionths of a second. Consequently, a PUV light system can produce very high peak-power pulses of light within a very short time, making it a sterilization tool that has been successfully used to kill bacteria and fungi in food products (Bialka et al. 2008) and water (Sharifi-Yazdi and Darghahi 2006). The killing effect is four- to sixfold higher than that of conventional continuous UV light treatment at the same energy level (MacGregor et al. 1998).

The wavelengths for UV processing in terms of effective microbial inactivation, termed the germicidal wavelength range, ranges from 200 to 280 nm. 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 damage occurs particularly between pyrimidine bases, resulting in an inhibition of replication and, in the case of lethal doses, in a loss of reproducibility. Two well-known types of mutagenic lesions in UV-irradiated DNA have been identified: (1) the formation of cyclobutane pyrimidine dimers (CPDs) between the C-4 and C-5 positions of adjacent thymidine or cytosine residues and (2) the formation of pyrimidine (6–4) photoproducts between the C6 and C4 position of adjacent pyrimidine residues, most often between T–C and C–C residues (Douki et al. 2003).

Microbes, however, possess several mechanisms to enable cell survival following UV exposure. One of these is reactivation. To a certain extent, DNA damage can be tolerated by the cell until repair occurs (Zimmer and Slawson 2002). The mechanism by which microorganisms recover replication activity is called photoactivation and occurs as a

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result of the direct reversion of thymine dimers (Douki et al. 2003). This process is catalysed by the DNA repair enzyme photolyase and requires visible light. In addition to photoreactivation, numerous light-independent repair mechanisms exist that are regulated by the expression of the single-strand DNA binding protein RecA (Makarova et al. 2001).

The aim of our study was to develop a DNA dosimeter method for monitoring the effectiveness of PUV light and to explore bacterial reactivation mechanisms (photoreactivation and dark repair).

Materials and methods

Bacterial strains The *Pseudomonas aeruginosa* strain used in this study was obtained from the American Type Culture Collection (ATCC 15442). Cultures were grown in Luria–Bertani broth (LB; in g/l: 10 tryptone, 5 yeast extract, 10 NaCl) or on LB solidified with 15 g/l agar (LBA). Saline [0.85 % (wt/vol) NaCl] was used for cell suspensions during UV irradiation.

PUV radiation The PUV system is based on the combination of power and flash UV lamp technology. PUV light differs from traditional continuous UV light by a much higher irradiance of UV illumination and a reduced exposure time. PUV flash-lamps commonly operate at pulse lengths ranging from a few tenths of milliseconds to longer than milliseconds. UV irradiation from a polychromatic UV source (UV-pulse lamp) was measured using a potassium iodide/iodate actinometry (KI/KIO_3) method according to Rahn et al. (2003). For this study, the UV dose determined by this chemical actinometry method was equal to 5.72 mJ/cm^2 per UV pulse. In order to reduce the photothermal effect of PUV light due to visible light and infra-red, the PUV system was equipped with a ventilator.

UV-irradiated bacteria To study the dose/response relationship and reactivation, we cultured the *P. aeruginosa* strain in LB broth. The bacterial suspension was diluted in phosphate buffered saline (PBS) in order to obtain a concentration in the range of 1×10^5 to 1×10^6 CFU/ml. prior to being used for the irradiation experiments. A 20-ml volume of the prepared suspensions was transferred into a standard petri dish for the eventual exposure to an increasing number of UV-light pulses.

Viable cell counts Viable cell counts were taken before and immediately after UV exposure. A 100-µl portion of each irradiated sample 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 solid LB medium. The number of colony-forming units (CFU/ml) or the 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).

Reactivation conditions After removing an initial aliquot to determine inactivation, the remaining UV-irradiated samples were divided into two portions which were then transferred onto two separate sterile petri dishes containing LB medium. One of the two petri dishes was exposed to visible light to examine potential photorepair and one was covered with foil to allow for potential dark repair at room temperature.

DNA extraction from P. aeruginosa Genomic DNA of *P. aeruginosa* was extracted immediately before and after irradiation by different doses of UV-C light, as well as after predetermined rest times using the 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, Hercules, CA) and analysed (Molecular Analyst software; Bio-Rad).

PCR conditions For 16S ribosomal DNA (rDNA) amplification, we used the universal bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 905R (5′-CCGTCAATTCATTTGAG-3′) primers (Kasuga et al. 2007). The 5′ end of the forward primer (27F) was labelled 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 cycling conditions consisted of an 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, with a final extension at 72 °C for 10 min. The amplified rDNA were quantified using a NanoDop® ND-1000 spectrophotometer (NanoDop Technologies, Wilmington, DE).

Terminal restriction fragment length polymorphism analysis The triplicate PCR products for each irradiated sample were mixed and purified using a MinElute PCR Purification kit (Qiagen, Hilden, Germany). The DNA concentration was quantified using a NanoDop® ND-1000 spectrophotometer (NanoDop Technologies). Restriction enzyme digestion was conducted in triplicate. The PCR products were digested with 10 U of the tetrameric restriction enzyme *Hha*I (TaKaRa BIO, Otsu, Japan) in a 20-µl volume according to the manufacturer's instructions. The digested products

were purified using a QIAquick Nucleotide Removal kit (Qiagen). The 6-FAM-labelled fragments were analysed with an ABI Prism® 310 Genetic Analyser (Applied Biosystems), and fragment analysis was carried out using GeneMapper™ v3.0 software (Applied Biosystems). The detection threshold for terminal-restriction fragments (T-RF) was set to 100 relative fluorescent units (RFU) for the software. The relative abundance of T-RFs was calculated based on their peak area.

Results and Discussion

UV dose–response

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

The lethal effects of the light pulses can be attributed to their rich broad-spectrum UV content, short duration and high peak power, all of which play a major role in bacterial inactivation (Sharifi-Yazdi and Darghahi 2006). 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 added to the PUV system to remove the UV wavelength region below 320 nm (Takeshita et al. 2003).

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

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

where N_0 is the number of viable cultivable bacterial cells before exposure to UV light, N is the number of viable cultivable bacterial cells after exposure to PUV light, A is a constant corresponding to bacteria retaining viability following UV irradiation, K is the coefficient of lethality, I is UV-C intensity (expressed in mW/cm^2), τ is the number of UV pulses and n is the 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.

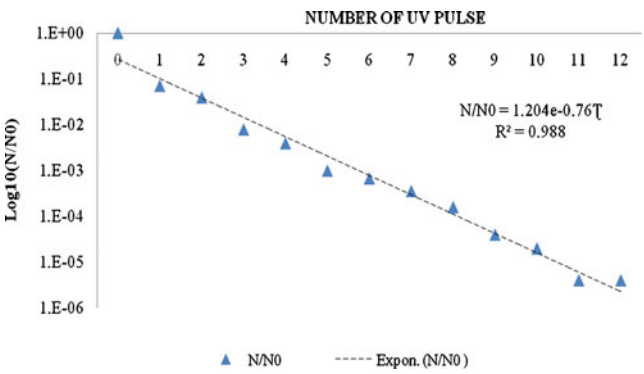

Fig. 1 The kinetics of *Pseudomonas aeruginosa* ATCC 15442 inactivation following exposure to ultraviolet-C (UV-C) radiation according to the model of Chick–Watson (for a full description of the parameters, see section UV dose–response). Where error bars are not shown, no differences between duplicates were detected

The inactivation kinetic (dose/response) according to the model of Chick–Watson (Eq. 1) shows that the irradiation of *P. aeruginosa* by 8 UV pulses is sufficient for 99.99 % inactivation of the colony-forming ability, which corresponds to a UV dose of 45.76 mJ/cm^2. This UV dose is nearest to the UV fluency generally used in Europe and the USA for the disinfection of drinking water. According to the literature, 40 mJ/cm^2 is sufficient to inactivate 4 unit-log$_{10}$ of such pathogenic bacteria as *Legionella*, enteric viruses, *Cryptosporidium* oocysts and *Giardia* cysts (US-EPA 2003).

Based on our analysis of the 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. However, this led to the question of whether the UV dose equivalent to 8 UV pulses is effective or not for inactivating bacteria at molecular level. To answer this question and to predict the biological effectiveness of the applied UV doses, we used a DNA dosimeter system based on 16S rDNA PCR amplification and terminal restriction fragment length polymorphism (T-RFLP) analysis to monitor the effects of PUV radiation.

DNA-dosimeter analyzed by PCR

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

Efforts are being made worldwide to develop dosimetric systems based on biological material that can be used to evaluate the biological effects of PUV radiation. Since DNA is the primary UV target in living organisms, DNA would appear to be the logical biological material or use as a molecular dosimeter for the detection of damage. Photoinduced DNA damage blocks synthesis during PCR assays, reducing the amount of amplified products of UV-exposed DNA compared to the control DNA.

In our study, the DNA-dosimeter system was obtained by the analysis of 16S rDNA PCR products for the same tested bacteria and under different irradiation conditions using 27F

and 905R primers. The amplified fragments were to be approximately 1,500 base pairs long (Fig. 2).

PCR inhibition was detected by agarose gel electrophoresis prior to T-RFLP analysis to check the size of the PCR products (Fig. 2). An intense band was visible for the unirradiated sample and irradiated samples with a low-intensity UV pulse. 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) exert continual disruption of PCR amplification as a function of increases in the number of UV light pulses.

Comparison between UV dose/response and DNA dosimeter

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

The average PUV effect was obtained by the equation –ln $A/(A_0)$, where A is the amount of amplified products with PUV-exposed DNA and A_0 is the amount of the amplified products of amplified unirradiated DNA (control test) (Fig. 3).

According to our 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 21 % of 16S rDNA amplification in vitro by PCR using 27F and 905R primers and Taq polymerase for DNA extension (Fig. 3). Despite the partially inhibition of PCR amplification, nearly 79 % of amplified 16S rDNA can be ensured in vitro. This percentage reveals that the equivalent dose of 8 UV pulses results in the inhibition of 99.99 % of bacterial cultivability in standard media, but not DNA replication and, consequently, not bacterial viability and toxicity.

We can therefore conclude that the limited information obtained from the simple count of viable and cultivable bacteria is incomplete. Indeed, some bacteria lose their cultivability property on appropriate growth media but can exhibit signs of metabolic activity and thus viability (Armisen and Servais 2004). The presence of these viable but non-culturable (VBNC) bacteria in the natural environment could be important from a public health point of view as some authors (Pommepuy et al. 1996; Servais et al. 2009; Ben Said et al. 2010) have suggested that pathogenic VBNC bacteria can maintain their virulence, thereby functioning as a potential reservoir of disease.

In addition, after irradiation by 12 UV pulses (approx. 68 mJ/cm^2), nearly 58 % of the 16S rDNA could be amplified (Fig. 3). This percentage shows the ability of VBNC bacteria not yet reactivated to ensure DNA replication and resuscitation. However, when the number of UV pulses was increased to >12 pulses, there was a significant inhibition of PCR amplification in terms of the accumulation of photoproducts generated by germicidal wavelengths of the PUV light. UV-induced DNA lesions, such as CPDs, have differential effects on DNA conformation, impairing their regulatory functions and other dynamic processes. These UV-DNA effects have a repercussion on DNA replication in vitro when assayed using PCR. Thus, an increasing number of UV light pulses can cause mutations in the primer binding sites on the template strand or a blockage of extension step assumed by Taq polymerase. It should be noted that the inhibition of rDNA amplification for a post-irradiated strain in vitro is similar to what is going in vivo at the bacterial DNA level during replication and transcription.

Bacterial reactivation

Figure 4 shows reactivation of *P. aeruginosa*-irradiated samples after a rest time in visible light and in darkness, respectively.

To semi-quantify the reactivation level of post-irradiated bacteria in visible light and/or in darkness, a log ratio was determined according to a modified version of the Lindauer and Darb equation (1994):

$$\text{Coefficient of reactivation } (C_r) = \log A_R/A_T$$

where A_T is the amount of the amplified products with PUV-exposed DNA and A_R is the amount of the amplified products of amplified DNA after a rest time in visible light or darkness (Fig. 4). According to Lindauer and Darby (1994), the reported log values range from 1 to 3.4. When the C_r is <1, there is no reactivation or no PUV-DNA damage repair;

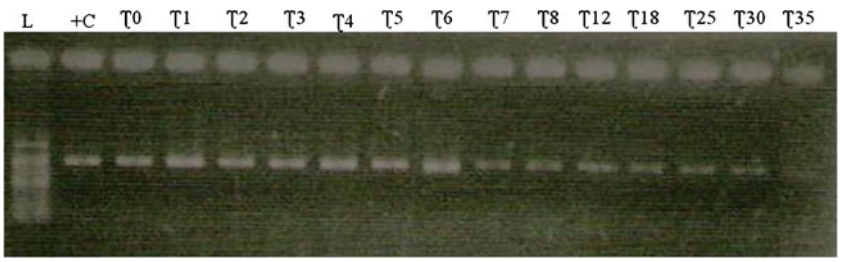

Fig. 2 Agarose gel electrophoresis of PCR products generated from irradiated *P. aeruginosa* with the primer set 27F and 905R. Image of a 1 % agarose gel stained with ethidium bromide. *Lanes*: *L* 100-bp ladder, *+C* positive control, T_n number of UV light pulses

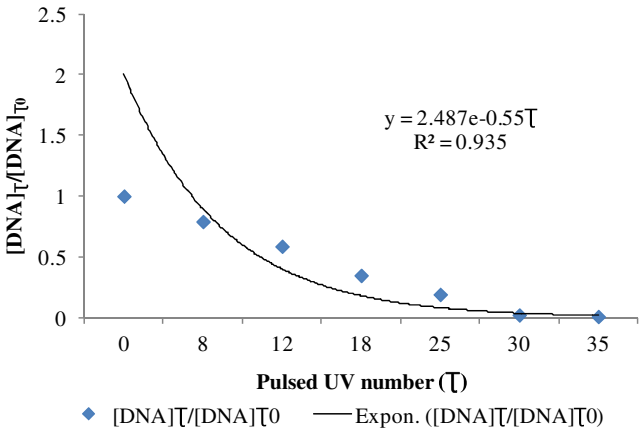

Fig. 3 DNA-dosimeter of *P. aeruginosa* using PCR amplification of 16S rDNA gene

at a C_r of between 1 and 3.4, we can conclude that reactivation occurs in the darkness or/and in visible light; when C_r is >3.4, there are no UV effects and the cells grow naturally without any environmental stress.

Figure 4 shows that the reactivation of post-irradiated *P. aeruginosa* is more important after a rest time in visible light than in darkness, especially after 8 and 12 pulses. Thus, the light-dependent repair (photoreactivation) was much more efficient than the light-independent mechanisms (dark repair) for restoring DNA replication in vitro as assayed by the PCR technique. However, after irradiation by a sub-lethal dose, we noted a regression in the number of PCR products as a function of exposure to UV dose both in visible light and in darkness.

DNA-dosimeter analysed by "peak-profiles T-RFLP"

A semi-quantitative molecular technique was developed for the rapid analysis of PUV light effects on rDNA amplification. The technique employed PCR in which one of the two primers contained a fluorescent label at the 5′ end and used 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 T-RF was precisely measured using an automated DNA sequencer (Kasuga et al. 2007).

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

Analysis of T-RFLP

Computer-simulated analysis of T-RFLP for UV-post-irradiated *P. aeruginosa* sequences showed that with the proper selection of PCR primers (27F and 905R) and restriction enzyme (*Hha*I), there is no difference in T-RF sizes. Indeed, all profiles consisted of a single identical T-RF of nearly 148 pb (±1 pb), although some of the T-RF had a different peak area. Relative peak area (RPA) was calculated as a percentage by dividing the 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 RPA or relative peak height is given in Fig. 5. 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 directly related to the number of UV light pulses applied (Fig. 5).

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

$$RPA_T / RPA_{T0} = A_{CPD} \exp(-k_i T) \quad (2)$$

where RPA_{T0} is the RPA calculated at time zero before UV irradiation, RPA_T is the RPA calculated after irradiation by a number (T) of UV light pulses, k_I is the inhibition

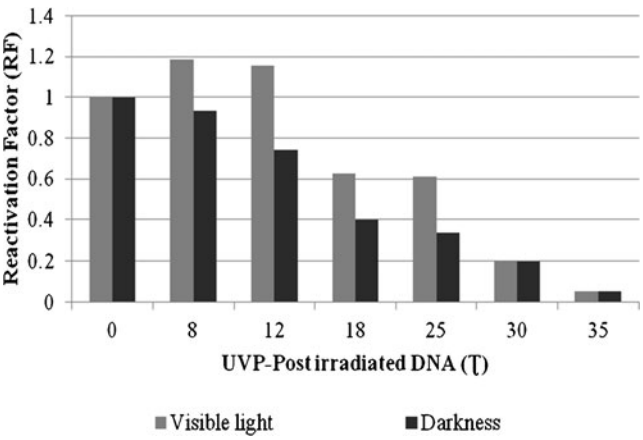

Fig. 4 Log increase of post-irradiated *P. aeruginosa* samples after a rest time in visible light and darkness, respectively. *PUV* Pulsed UV light

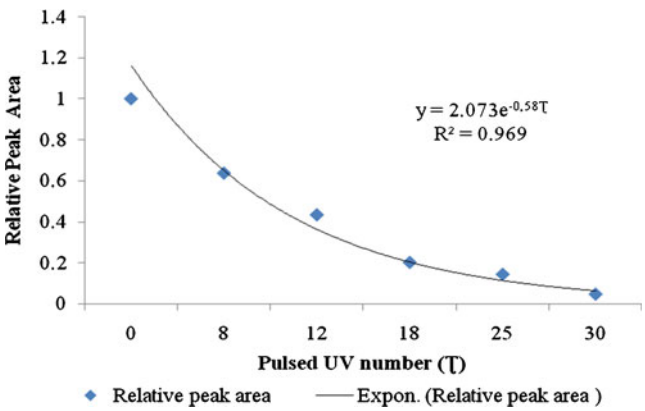

Fig. 5 Relative peak area of terminal-restriction fragments of irradiated *P. aeruginosa* ATCC 15442 in relation to an increasing number of UV light pulses

coefficient of a specific T-RF and A_{CPD} is the photoproduct accumulation rate.

The T-RFLP technique is based on the determination of the RPA of the T-RF generated by a restriction enzyme after the PCR step. Consequently, T-RFLP analysis allows us to focus on the effects of PUV on bacterial DNA. In addition, after irradiation by PUV light, there was a decrease in the RPA of the specific T-RF for irradiated DNA (Fig. 5). For example,, after irradiation by 8 pulses of UV light and an inactivation of 99.99 % of viable and cultivable bacteria, the RPA of T-RF is equal to 64 % compared to the RPA determined for *P. aeruginosa* at time zero before UV irradiation. Moreover, after 12 UV pulses, the RPA_T (%) is equal to 43 % of the single T-RF compared to RPA_{T0} at time zero before UV irradiation. According to the inactivation kinetic and reactivation study of tested bacteria (Fig. 1), this applied dose would cause the loss of bacteria cultivability in standard media with subsequent reactivation.

Also, the persistence of a specific T-RF despite the increasing irradiation by PUV light shows a higher intrinsic resistance of studied *P. aeruginosa* against UV irradiation. The T-RF was found to disappear after 30 UV pulses.

The relative abundance of bacteria in the irradiated samples, as shown by our DNA-dosimetry system, strengthens the argument that different "bacterial viability forms" exist among the same irradiated bacteria. Indeed, the single T-RF can probably include VBNC bacteria not yet reactivated (after 12 UV pulses), active but non-cultivable bacteria (after 18 UV pulses) and VBNC-UV-inactivated bacteria (after sublethal UV dose). This possibility is 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 sterilization/disinfection treatment of water has been shown to be useful.

Conclusion

The public health risk from bacterial infection is not only a function of the abundance of the microorganism's contaminants in water but also of their capacity to survive in the treated environment and maintain their virulence (Ben said et al. 2010). Therefore, the disinfection system process needs to be effective and accurate and reliable techniques, such as molecular methods, need to be developed to compare the survival of the bacteria upstream and downstream of the disinfection system and to study the infectivity and the virulence of the microorganisms treated by UV light (continuous UV radiation or PUV light).

The DNA-dosimetry based approach presented here 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 biodosimetry which is based only on the determination of viable and cultivable bacteria after UV treatment.

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