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Full Length Research Paper

Detection of active *Escherichia coli* after irradiation by pulsed UV light using a Qβ phage

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The infectivity rates of Qβ phage is used as a bio-indicator of the physiological state of host cells and for the detection of active *Escherichia coli* irradiated by an increasing number of pulsed ultra-violet (UV) light. Indeed, the combination of a conventional method used to measure colony-forming ability of tested bacteria after exposure to an increase number of pulsed UV light, and the simulation of phage adsorption kinetic, can reveal the existence of active bacteria which lose the cultivability in usual culture media, but keep viability and phage susceptibility.

Keys words: Ultra-violet inactivation, viable but non-culturable bacteria, Qβ phage, active bacteria.

INTRODUCTION

Disinfection, as applied in water and wastewater treatment, is a process by which pathogenic micro-organisms are inactivated to provide public health protection. Chlorination has been used for most water disinfection operation for many years. However, it is no longer the disinfection method automatically chosen for either water or wastewater treatment because of potential problems with disinfection by products and associated toxicity in treated water. Among the alternatives to conventional chlorination, ultra-violet (UV) irradiation is chosen the most frequently (Bolton et al., 2003). The effectiveness of 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 260 nm (Byrd et al., 1990). This absorption creates damage in the DNA by altering nucleotide base pairing, thereby creating new linkages between adjacent nucleotides on the same DNA strand. If the damage goes unrepaired, the accumulation of DNA photoproducts can be lethal to cells through the blockage of DNA replication and RNA transcription, which ultimately result in reproductive cell death (Zimmer and Slawson, 2002). However, UV disinfection is noted to have some problems, one of them is reactivation. Most bacteria repair these lesions in two ways; light dependent photoreactivation catalyzed by an enzyme name photolyase and light-independent restore mechanisms such as nucleotide excision repair (Liltved and Landfalde, 1996). The goal is the production of save water, thereby, after disinfection of water, health protection programs require to estimate the level of contamination of treated water (Byrd et al., 1990).

The methodologies used to evaluate the performance of disinfection system, is based on the determination of colony-forming ability of indicator bacteria after treatment. However, the information given from the simple viable and cultivable count of microorganisms is incomplete. Indeed, the investigations of bacterial survival in natural environments have indicated that some of their lose notion of cultivability on appropriate growth media under certain conditions and yet still exhibit signs of metabolic activity and thus viability (Armisen and Servais, 2004).

For years, total coliforms and fecal coliform were the most widely used indicators for water quality but, more

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recently, the abundance of *Escherichia coli* has been shown to be more related to the sanitary risk than that of coliforms (Armisen and Servais, 2004). Accurate enumeration of *E. coli* is thus important to assess microbiological water quality. Classical methods for enumerating *E. coli* are based on culture in liquid (most probable number, MPN) or solid (plate counts) media (Byrd et al., 1990). These methods are not allowed to detect all the target bacteria in natural environments. Indeed, when released in natural waters, fecal bacteria were shown to lose their ability to growth on culture media while preserving their viability.

The presence of these viable but non-culturable (VBNC) bacteria in the environment could be important from a sanitary point of view as some authors (Colwell et al., 1996; Pommepuy et al., 1996) suggested that pathogenic VBNC bacteria could maintain their virulence being thus a potential reservoir of disease. The public health risk is thus not an only function of the abundance of the microorganism's contaminants in water, but also of their capacity to survive in the receiving environments and to maintain their virulence (Chedad and Assobhei, 2007).

As a consequence, alternative methods were developed during the last 10 years to detect and enumerate *E. coli* in waters. Those are direct enzymatic methods, immunological methods, quantitative polymerrase chain reaction (PCR) and fluorescent in situ hybridization (FISH) (Armisen and Servais, 2004).

The purpose of this study is to determine the relationship between indicator of fecal indicator bacteria (*E. coli*) and phage (Qβ phage) in order to detect the presence of active bacteria undetected by culture in usual media.

MATERIALS AND METHODS

Bacterial strain and bacteriophage

The RNA F-specific coliphage, Qβ phage and its cell host, *E. coli*, were obtained from the American type culture collection (23631-B1 and 13965, respectively).

Qβ phage was commonly recommended for modelling viral behaviour in water (Espinosa et al., 2009) and also was used in biological actinometry (Biodosimetry) (Fallon et al., 2007). In this study, we used Qβ phage as an indicator of active VBNC bacteria after UV irradiation.

Pulsed UV radiation

The pulsed UV system is developed by the combination with power and flash UV lamp technology. Pulsed UV light was differed from the traditional continuous UV light by much higher irradiance of UV illumination and reduction of exposure time. Indeed a flash lamps commonly use in operating 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 Potassium 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. Noting that, in order to reduce the photo-thermal effect of pulsed UV light due to visible light and infra-red, the pulsed UV system was equipped by a ventilator.

UV-irradiated bacteria

For dose/response relationship and reactivation experiments, the strain of *E. coli* was cultured in Luria-Bertani broth (LB). Bacterial suspension was diluted in saline Phosphate buffers (PBS) in order to obtain a concentration ranged from 1 x 10^5 to 1 x 10^6 bacteria per 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 pulsed UV-light (0, 8, 12, 18, 25 and 30 UV pulses).

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).

Qβ phage replication experiments

To investigate the presence of active bacteria in the UV-irradiated sample, Qβ phage adsorption experiments were performed with a modification of the procedure of Woody and Cliver (1995). UV-irradiated suspensions of host cells were infected with Qβ phage. After infection and at the indicated time intervals (each 5 min after infection), 100 µl was removed from different suspensions and after a cumulative dilution, a volume of 100 µl of each dilution was periodically assayed to determine the phage adsorption's kinetic to the host cell. The phage titration was determined using the double-layer agar plate method. Qβ phage replication experiments were determined at time zero, before UV irradiation; using initial host cells and phage densities to know the optimal timing of phage adsorption.

In this work, we have repeated all experiments more than 3 times and we have choice to work with average values. This choice was based on the use of different mathematical models to simulate the Qβ phage's replication and to determine different kinetic parameters related to the phage adsorption to the host cells under different UV irradiation conditions.

Simulating Qβ phage's growth

To simulate Qβ phage adsorption to the host cells, using the model employed by Levin et al. (1977) and reported by Abedon (2001). The modelling of Qβ phage adsorption was applied to investigate the impact of host cell after irradiation by an increasing number of pulsed lights, in the phage adsorption kinetic. The adsorption constant (k) and the density of uninfected host cells (N') were determined and compared to control test determined at time zero using initial cell and phage densities. In addition, the adsorption time (t_A) was determined according to Wang et al. (1996).

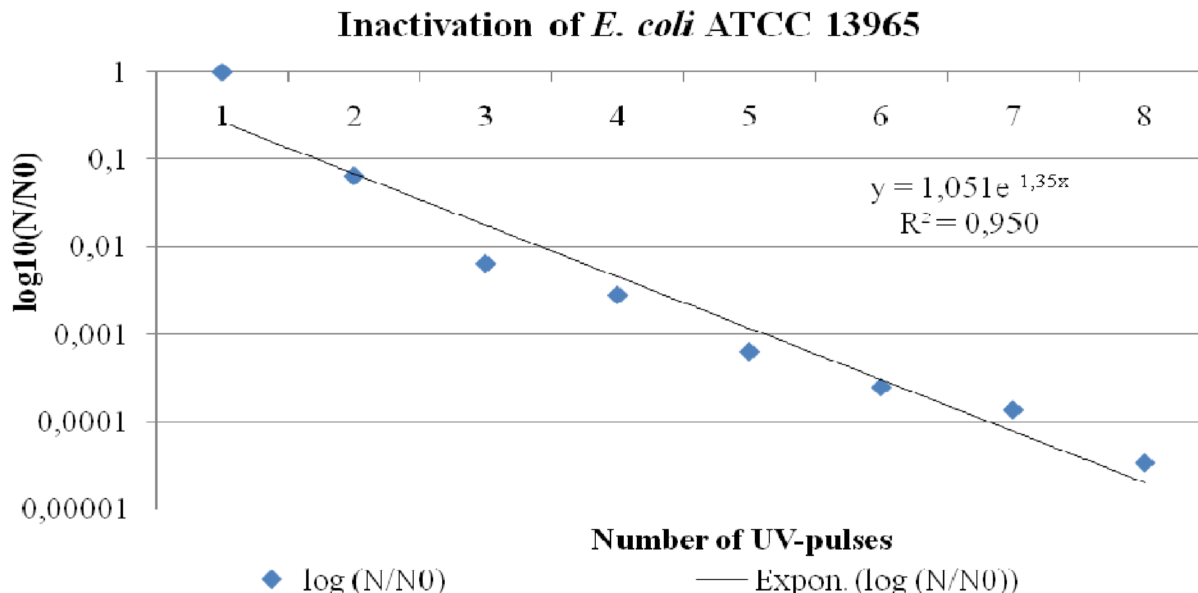

Figure 1. The Kinetic of *E. coli* ATCC13965 inactivation following exposure to UV-C irradiation according to the model of Chick-Watson, *y*: Reduction = N/N_0 with N_0: Number of viable and cultivable cell before exposure to UV light; *N*: Number of viable and cultivable cell after exposure to UV-C irradiation, x = $I^n t$ with *I*: UV intensity (mW/cm^2), *t*: exposition time (s), *n*: Threshold level of series-event model, *n* = 1 for the first order Chick-Watson model, where error bar are not shown, differences between duplicates were not detected.

Titration of Qβ phage

The irradiated host cell respectively by 0, 8, 12, 18, 25 and 30 UV pulses infected Qβ phage were incubated at 37°C. After 18 h, all cell-phage suspensions were filter sterilized using a 0.45 µm syringe filter to obtain free phage in the filtrate. After that, the supernatants were diluted in order to determine the titration of phage. The phage titre was determined by using a standard double-layer agar plate. After appropriate dilution with PBS buffer, a volume equal to 100 µl of free phage was added to a 100 µl of active growing culture of *E. coli* (10^6 CFU/ml) and the mix were added to 3 ml of soft agar (maintained at 48°C). The mixture was overlaid on TSA agar plates and incubated 18 h at 37°C to enumerate plaques.

The phage titration experiment was determined at time zero using initial unirradiated cells and phage densities. All phage adsorption' experiments were done more than three times, to verify the sensibility and the reproducibility of this method.

RESULTS AND DISCUSSION

The inactivation kinetic of *E. coli* ATCC13965: Pulsed UV dose/response

The inactivation kinetic of *E. coli* ATCC 13965 was function of UV-C dose. The germicidal dose was expressed as the product of UV radiation intensity (*I*) and number of pulsed UV light (T) (Figure 1). 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).

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

Where, N_0 is Number of viable cultivable bacteria before exposure to UV light; *N* is Number of viable cultivable bacteria after exposure to pulsed UV light, *A* is constant corresponding to bacteria retaining viability following UV irradiation, *k* is Coefficient of lethality, *I* is the UV-C intensity expressed in mW/cm^2, T is number of UV pulse and *n* is threshold level of series-event modem, *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 shows that the irradiation of *E. coli* by 8 UV pulses is sufficient to inactivation 99.99% of colony-forming ability thus, after exposure to a UV dose equal to 45.76 mJ/cm^2. This UV dose is nearest of the UV fluency used usually in Europe and USA for the disinfection of drinking water. Indeed, according to the literature, 40 mJ/cm^2 is enough to inactivate 4 U-$\log_{10}$ of pathogenic bacteria as *Legionella*, enteric viruses, *Cryptosporidium* oocysts and *Giardia* cysts (US-EPA, 2003).

Exploitation of VBNC bacteria after UV irradiation

According to the inactivation kinetic, *E. coli* loss the colony forming ability after irradiation by 12 pulses of UV light (non detected CFU/ml; ND). But it is well known that the loss of bacterial cultivability is not synonyms of

bacterial death. The question is how we can detect the presence of active but non cultivable bacteria in irradiated samples?

Detection of active post-UV-irradiated *E. coli* ATCC 13965: Study of Qβ phage replication in presence of VBNC host cell

Our propose is to detect the presence of VBNC *E. coli* after UV irradiation and then measured its production of Qβ phage during a single cycle of infection.

The lytic Qβ phage life cycle involves free-phage diffusion, host cell adsorption, an eclipse period, a period of progeny maturation and host cell lysis (Abedon et al., 2003). Lysis ends the phage latent period but initiates the extracellular diffusion of phage progeny to new host cells. The aim of this study was to examine the potential replication of Qβ phage in relation with UV irradiated *E. coli* (host cell) not detected in usual growth media. Based on UV-inactivation's kinetic curve of *E. coli*, we are exposed the tested bacteria to 8, 12 and 18 UV pulses.

These doses allowed respectively the inactivation of 99.99% of bacteria; the loss of cultivability of *E. coli* with and without subsequent reactivation. Moreover, exposure of the host bacteria to a higher number of UV pulse (25 and 30 UV pulses) in order to verify the sensibility of lytic phage to detect active bacteria persist in the irradiated suspensions.This series of experiments were conducted to determine in part, the detection of active bacteria present in the UV irradiated suspensions and on the another hand, to reveal the capability of VBNC bacteria to support phage replication.

Study of phage adsorption kinetic

Data exposed in Figure 2, do not show an inhibitory effects on the first step of phage replication in relationship with an irradiated bacteria. This result demonstrates the presence of enough bacteria in good physiological condition despite, the UV irradiation conditions and the decrease of viable and cultivable bacteria density. The modeling of phage adsorption was determined according the model employed by Levin et al (1977):

$$P(t) = P_0\, e^{-k'.N'.t} = P_0.\,(1- k'.\, N'.\, t)$$

Where; P_0 is the initiated free-phage concentration at time Zero, (t_0); $P(t)$ is the free-phage concentration at time (t); k' is the phage adsorption constant, and N' is the density of uninfected host cells.

The adsorption constant K', determined by the analysis of the adsorption curves (Figure 2), showed that the level of phage adsorption to host cells decrease with the increase number of pulsed UV light. The changes in the phage growth parameters (adsorption phage, elongation of latent period) were probably due of the changes of host quality (stressed and damaged bacteria by UV light in poorer growth environment, PBS buffer).

For instance, after inactivation of 99.99% of host bacteria, the constants of Qβ phage replication's kinetic, do not affect compared with the control test (phage in the presence of unirradiated host cell). Although, the phage adsorption rate, determined after irradiation of host cell by 25 and 30 UV pulses is lower than 6.2 to 8 fold respectively compared to the adsorption constant determined at time zero, before UV irradiation. In this case, the decrease of phage adsorption rate can be explained by the fact that, the exposure of host bacteria to a sub lethal dose can conduct to a modification of a physiological and metabolic state of irradiated bacteria cell caused by the bactericidal effect of UV light (Makarova et al., 2000).

Furthermore, it is well known that the phage adsorption rates are proportional to host cell surface area (Woody and Cliver, 1996); thereby, after UV irradiation, bacteria can change its morphological shape as a strategy of bio-protection against stress and to survive in a hostile environment (Langer and Hengge-Aronis, 1991). In our case, to escape or to minimize the germicidal effect of UV irradiation, bacteria has changed its shape consequently; this change can have an effect on the phage adsorption by stopping or prolonging the timing of phage adsorption to the host cell.

Determination of uninfected bacteria

After fixing the timing of optimal phage adsorption at 45 min based on the interaction of initial cell density and Qβ phage (at time zero before UV radiation), it was possible to achieve reasonably simulation of the number of uninfected host cells after each irradiated by an increase UV dose samples according to the model of Levin et al (1977). Time adsorption (t_A) was defined as the mean free time (MFT) according to Wang et al (1996): $t_A = (k'N')^{-1}$

The MFT represents the average length of time a cohort of free phages requires to adsorb to host cells expressed by min/host cell (Table 1). Noted that, 15 min was chosen to determine the time of the initial uninfected host cells density and this host cells density was fixed as an initial density of viable but non cultivable bacteria (VBNC) infected by a constant amount of phage.

The uninfected cell density can reflect directly the level of inactivated bacteria by a germicidal effect of UV light compared with time zero before UV irradiation (maximum level of phage infectivity). In fact, when we increase the UV exposure dose, the density of uninfected bacteria increases.

The results exposed in Table 1 shown, that the time required for the Qβ phage to be attached in the host cell increase, with the increase of UV dose. This result, support the previous results concerning the decrease of adsorption kinetic constant (k') and the increase of

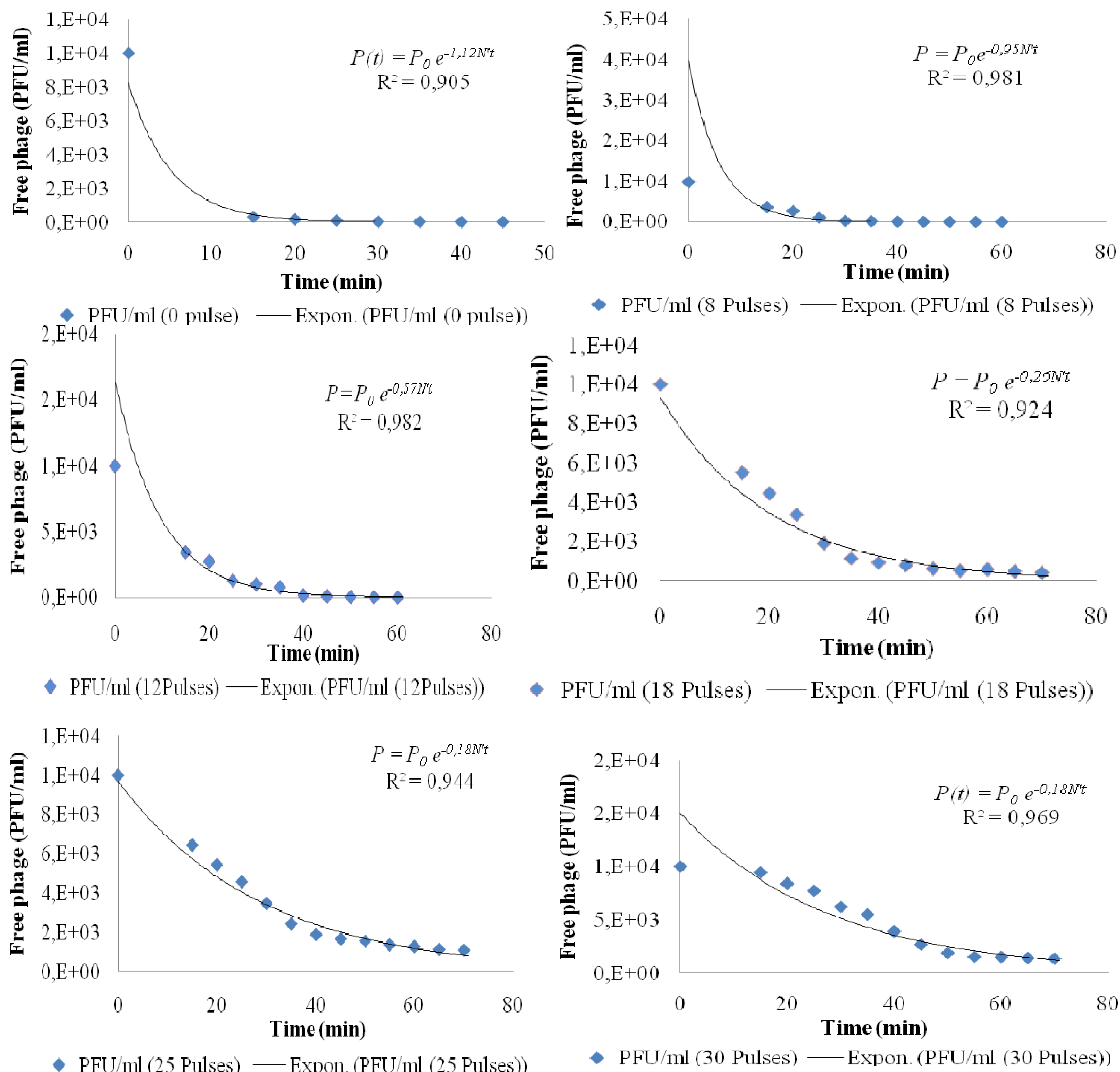

Figure 2. Qβ phage adsorption kinetics dependence on host UV irradiation state (UV irradiation by 0, 8, 12, 18, 25 and 30 UV pulses), P_0: the initiated free-phage concentration at time zero (t_0), $P(t)$: the free-phage concentration at time (t), K': the phage adsorption constant and N': the density of uninfected host cells.

Table 1. Determination of different parameters related to Qβ phage growth kinetic

	Number of UV Pulses					
	0	**8**	**12**	**18**	**25**	**30**
Qβ Phage adsorption constant (K')	1.12	0.95	0.57	0.26	0.18	0.14
Density of uninfected host cells (N')	0.019	0.02	0.04	0.09	0.12	0.13
Adsorption time (t_A)	0.04	0.07	0.20	0.98	2.04	2.57

Legend: K': Phage adsorption constant (ml/min); N': Number of uninfected cells at time 15 min (cell/ml); t_A: Adsorption time (min/host cell).

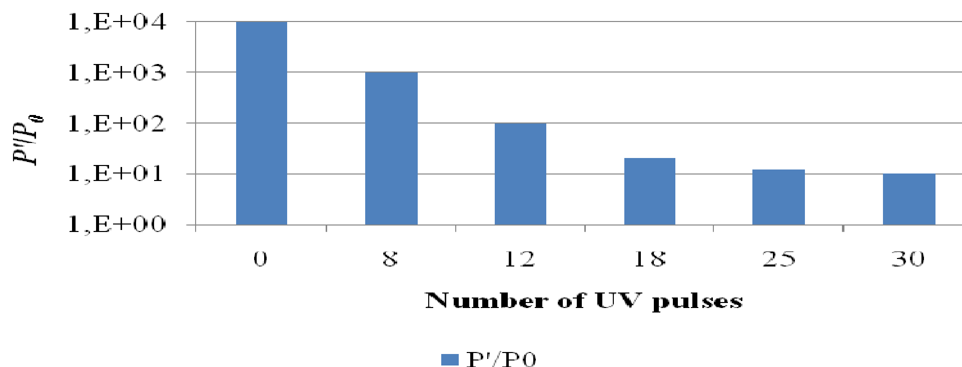

Figure 3. Qβ phage titers dependence on host UV irradiation state after 18 h of incubation at 37°C, P_0: the Qβ phage titter after incubation in the presence of unirradiated bacteria, *P'*: the Qβ phage titres after 18 h of incubation at 37°C in the presence of irradiated *E. coli* by 8, 12, 18, 25 and 30 pulsed UV light .

uninfected hosts cells. In fact, there changes in the phage growth parameters were caused by the change in host quality and therefore by the pressure of selection of good quality of host cells by phage (Wang et al., 1996). Conclusively UV-irradiation of host cells have a negatively affect phage growth, by lengthening the phage latent period, or reducing the phage adsorption constants.

The titration of Qβ phage, after 18 h of incubation at 37°C in the present of susceptible host cells after UV irradiation by increasing number of pulsed UV light; showed an increase of phage density. Compared to the control test (infection of non irradiated host cell by an initial concentration of Qβ phage; P_0), different level of progeny phage was release in relation with UV *E. coli* irradiation conditions.

The amount of phages released by irradiated host cell by 8 pulsed UV light is higher than the amount of phages released by *E. coli* irradiated respectively by 12, 18, 25 and 30 pulsed UV light (Figure 3). The decrease in the level of phage infectivity and release of infectious Qβ progeny phage is directly related by the decrease of active bacteria's density in relation with the increase of exposure of UV dose. Figure 3 shown, that the infection of irradiated *E. coli* by Qβ phage allowed the detection of active cells. In fact, despite their loss of bacteria's cultivability in usual growth media and the affection of phage replication cycle (by extension of latent period, decrease in the adsorption constant, etc.); portion ofVBNC bacteria or in the correct term, active bacteria Can let a replication of phage manifested by the enhance of the phage' titers after 18 h of incubation at 37°C.

The increase in bacteriophage titer and the maintenance of infectivity of VBNC bacteria or active but non cultivable bacteria can be related by the sigma factor RpoS. In fact, RpoS was shown to have a key role in survival of bacterial cells exposed to starvation, freezing, desiccation and UV irradiation, in addition to its established role in oxidative and osmotic stress response (Saint-Ruf et al., 2004; Hengge-Aronis., 2002).

The activation of *rpoS*-encoded σ^s subunit of RNA polymerase in *E. coli* is a global regulatory factor involved in several stress responses. HF-I protein is a component that, is essential for *rpoS* translation. This factor is encoded by the *hfq* gene (Muffler et al., 1996). This gene, *hfq*-encoded RNA-binding protein HF-I, which has been known previously only as a host factor for the replication of phage Qβ RNA; as an essential factor for *rpoS* translation (Muffler et al., 1996).

HF-I constitute a part of the Qβ replicase and was required for the synthesis of the minus strand from the original viral RNA (Barrera et al., 1993). Based on this information, hypothesis was establish as follows: the enhancement of Qβ phage titer was related probably by the induction of *hfq* gene and consequently, the increase of the intracellular concentration in HF-I factor in relation with the induction of alternative sigma factor (RspoS) involved in bacteria stress response.

In the end of this study, conclusion have be made that different phage growth constants defining phage-host cell interaction (extension of latent period, change in the timing of phage adsorption to the host cell, etc.) were directly related by the host cell quality (damaged cells by UV light, accumulation of photoproduct, enter in a VBNC state, etc).

The infectivity rates of Qβ phage is used as a bio-captor or bio-indicator of the physiological state of irradiated host cell mainly, the active but non cultivable bacteria. Thus, we can use this propriety to evaluate water after disinfection step by physical or chemical process and also to control food safety.

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