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Full Length Research Paper

Effect of ultraviolet, electromagnetic radiation subtype C (UV-C) dose on biofilm formation by *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa **is a commonly found nosocomial opportunistic pathogen that is characterized by its ability to form biofilm to protect itself from various external pressures. Biofilms are problematic in particular food industry sectors and in water distribution systems. In this study, we propose to explore the impact of UVC dose on biofilm production by *P. aeruginosa* strains. The biofilms densities were assessed by crystal violet assay. A relationship between UVC exposed dose and biofilm density was examined by establishment of new model linking between UVC dose and physiology bacteria response concerning biofilm production (UVC dose/ biofilm production).**

Key words: UVC dose, biofilm, *P. aeruginosa*, quorum sensing, RecA, model.

INTRODUCTION

Microorganisms are naturally tendency to attach to wet surfaces, to multiply and to embed themselves in a slimy matrix composed of extracellular polymeric substances (EPS) that they produce, forming a resistance structure called, biofilm (Simoes et al., 2010).

The resistance properties of biofilm communities are thought to be a function of multiple factors including the physical structure of the biofilm, cellular growth status, general stress response and resistant subpopulations of cells (Marques et al., 2005).

Various stages were including for bacterial biofilm formation. Microscopic analysis has indicated that biofilm formation occurs in a sequential process of (i) transport of bacteria to a surface; (ii) initial attachment; (iii) formation of micro-colonies; and (iv) biofilm maturation (O'Toole et al., 2000Sauer et al., 2002).

Biofilms are problematic in particular food industry sectors (Somers and Wong, 2004). In addition, biofilms are ubiquitous on surfaces in drinking-water distribution systems, Drinking-water biofilms are formed predominantly by heterotrophic microorganisms of the autochthonous aquatic microflora without any relevance to human health. Occasionally, biofilms can act as a reservoir for microorganisms with pathogenic properties (Rivardo et al., 2009). When these organisms persist and multiply within biofilms, and are released from the biofilms into the water phase, they result in the deterioration of the hygienic quality of drinking water and pose a potential threat to human health (Simoes et al., 2010).

It is well known that bioflms protect microbes against disinfectants (Momba and Binda., 2002) The accumulation of micro-organisms on the surface and the formation of bioflms depend on many factors prevailing in the water system. These include types of surface materials, disinfectant, concentration and quality of nutrients, the microbial quality of intake water, temperature, the hydraulics of the system, the age of the

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Abbreviations: **H_s,** threshold UVC dose (mJ.cm^{-2}); **H_t,** UVC dose over than *Hs* dose (mJ.cm^{-2}); **M_a,** Active bacteria responsible for production and maturation of biofilm; **M_b**, The total biomass of the biofilm continues to grow for the control test (before exposure to UVC light; H_{UV}=0 mJ.cm^{-2}); **M_d**, Inactive bacteria no longer involved in the construction of biofilm; ***P. aeruginosa,*** *Pseudomonas aeruginosa;* **QS,** quorum sensing; **UV radiation,** Ultraviolet radiation; **µ,** Biofilm growth rate.

water in the distribution system and, above all, the presence of a disinfectant residual (Momba et al., 1998). Consequently, the selection of water disinfection process was primordial to prevent the biofilm formation and 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 (Gallard and Von Gunten, 2002). Among the alternatives to conventional chlorination, ultraviolet (UVC) irradiation is chosen the most frequently.

The effectiveness of UVC 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. This absorption creates damage in the DNA by altering nucleotide base pairing; thereby creating new linkages between adjacent nucleotides on the same DNA strand. This damage occurs particularly between pyrimidine bases (Ben Said et al., 2009).

P. aeruginosa is a Gram-negative bacterium nearly ubiquitous in the environment. It is found in freshwater, soils, and man-made water fixtures (particularly faucets and showers), and is the most prevalent antibiotic resistant, opportunistic pathogen of humans (Stover et al., 2000). The typical *Pseudomonas* bacterium in nature might be found in a biofilm, attached to some surface or substrate, or in a planktonic form, as a unicellular organism, actively swimming by means of its flagellum. *Pseudomonas* is one of the most vigorous, fast-swimming bacteria seen in hay infusions and pond water samples (Harunur and Kornberg, 2000). In addition*, P. aeruginosa* strains exhibit significant variability in pathogenicity and ecologicalflexibility. Such inter-strain differences reflect the dynamic nature of the *P. aeruginosa* genome, which is composed of a relatively invariable "core genome" and a highly variable "accessory genome" (Klausen et al., 2003; Kung et al., 2010).

In public drinking-water systems, *P. aeruginosa* can occur sporadically. Indeed, according to Bressler et al. (2009), this bacteria strain is able to persist in a drinking-water for extended periods and it is often difficult to eliminate from water systems. This persistence is due to the development of bacteria on biofilm as biological barrier against biocide.

To reduce the biofilm production, novel and interesting approaches were recently emerged for instance, the treat of biofilms with lytic phages. This approach is being used to treat *P. aeruginosa* biofilms in cystic fibrosis patients by spreading aerosol that contains the phages (Beckmann et al., 2005).

The question is: UVC irradiation can prevent or not the biofilm production by *P. aeruginosa*? We propose in this study to investigate the effect of exposure UVC dose on bacterial physiology response concerning the biofilm production by *P. aeruginosa* strains: UV dose / biofilm production relationship.

MATERIALS AND METHODS

Bacterial strains

P. aeruginosa ATCC 15422 (PA_{ref}) obtained from American Type Culture Collection was selected as the biofilms-forming organism in this study in addition to five *P. aeruginosa* strains isolated from wastewater (PA_1, PA_2, PA_3,PA_4 and PA_5).

UVC irradiation

For the study of UVC dose/biofilm production relationship, the strains of *P. aeruginosa* were cultured in Luria-Bertani broth (LB). Bacterial suspensions were diluted in saline Phosphate Buffer (PBS) and the resulting preparations were used for irradiation experiments. A volume of 20 ml of the suspended culture was transferred into a standard Petri dish for exposure to the continuous UV-light treatment. The samples were exposed to the UV-light for chosen increasing UV dose (0, 10, 20, 30, 40, 60, 80 and 100 $mJ.s.cm^{-2}$).

UV dose/ biofilm formation by *P. aeruginosa* strains

Biofilm formation was quantified as described (O'Toole and Kolter, 1998). In order to test different strains of *P. aeruginosa* for their ability to produce biofilm, an overnight culture in LB broth were diluted 100-fold in fresh LB both and 200 µl was added to each well of a 96 wells of Microtiter plate. After 24 h of incubation at 30°C, the cultures were stained by the addition of 25 µl of 1% Crystal Violet (CV) solution to each well for 15 min at room temperature.

For irradiated samples, the same protocol was done to test the response of tested bacteria to exposure UV dose. A volume of 200 µl of each irradiated time was added to wells of Microtiter plate. After incubation, the excess broth was removed, and biofilm were washed and stained with crystal violet.

Wells were rinsed repeatedly with phosphate-buffered saline three times to remove ant unbound cells. Biofilm formation was suspended with the addition of 200 µl of 95% ethanol to each CV stained well and transferring the entire ethanol suspension into a 1.5 ml eppendorf tube. The volume was raised to 1ml by the addition of 800 µl ethanol and the absorbance was measured spectrophotometrically at an optical density of 600 nm (OD_{600}).

Biofilm formation was determined by an increase in optical density compared to the media only control. Each sample was analyzed in triplicate.

RESULTS AND DISCUSSION

P. aeruginosa is a ubiquitous bacteria recognized by its genetic and metabolic flexibility. In addition, this strain of bacteria can expressed many virulence factors. These factors are either related or secreted by bacteria and they are involved in various stages of host infection (human, animal or plant).

In this study, we are adopted *P. aeruginosa* as a representative model for study of bacterial biofilm for the reason that *Pseudomonas aeruginosa* is a model organism for the study of bacterial social behaviour, or

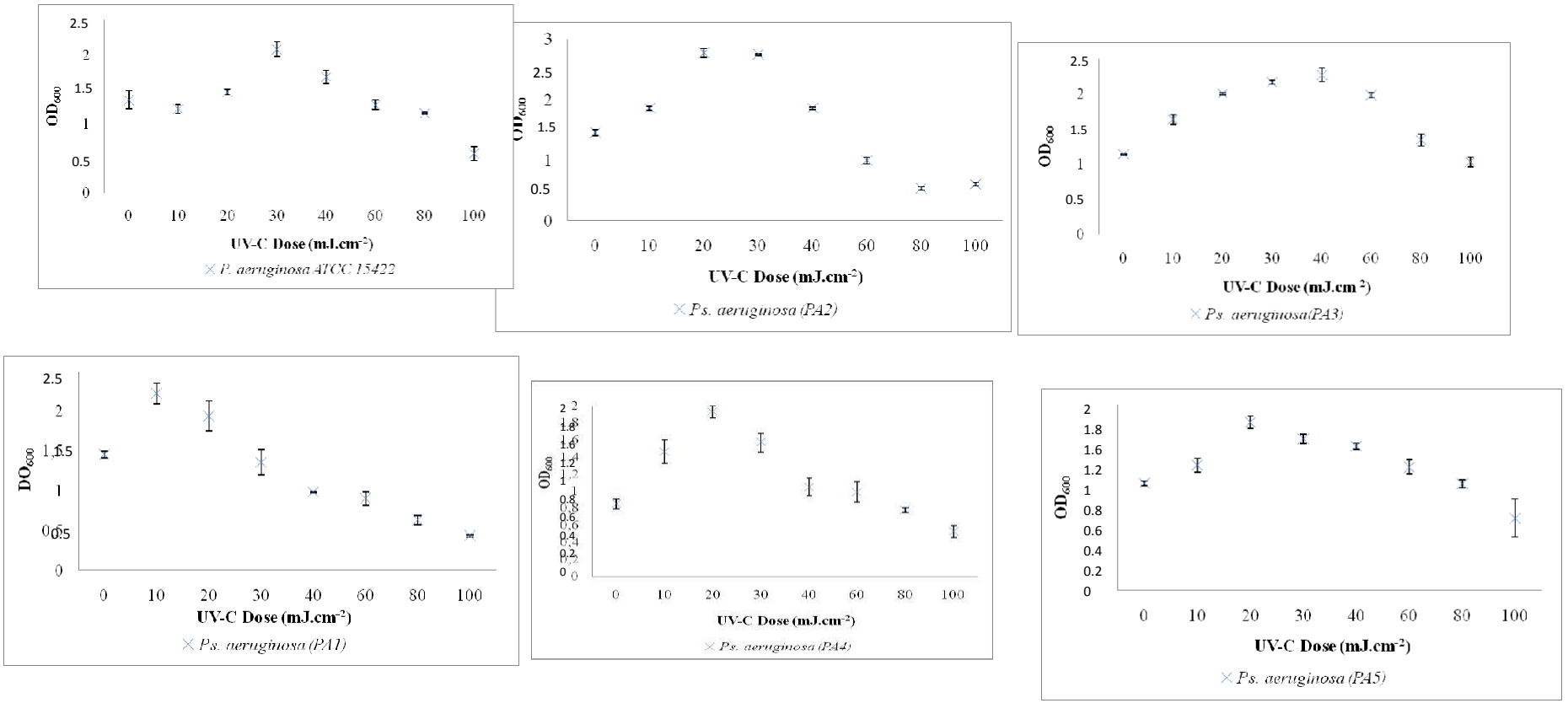

Figure 1. Study of relationship: UVC Dose / biofilm production by *P. aeruginosa* strains.

„sociomicrobiology" (Shrout et al., 2006). Indeed, from laboratory studies it is known that *P. aeruginosa* can form monospecies biofilms on metal, plastic and rubber materials (Bressler et al., 2009).

UV dose/ biofilm formation by *P. aeruginosa* strains

Biofilm formation appears to be a widespread attribute of bacteria and may allow increased survival ability under stressful conditions such as low nutrients or antimicrobial treatments (Mah and O"Toole, 2001).

The aim of this study was to evaluate the impact of UVC dose on biofilm formation by *P. aeruginosa* strains. In another word; we were interested to study the response of bacteria concerning the biofilm formation after exposure to a determined UV dose (H_{UV}).

After irradiation of *P. aeruginosa* strains by an increasing UVC dose: 0, 10, 20, 30, 40, 60, 80 and 100 mJ.cm $^{-2}$, we were tested the potential of bacterial strains to product biofilm using the method described by O'Toole et al. (1998) (Figure 1). Known that the UV dose equal to 40 mJ.cm^{-2} is the dose recommended for the disinfection of water in Europe and America (US-EPA 2003) Figure 1 shown that, for the majority of tested bacteria strains, the biofilm production presents a progressive increase in function of an increasing of exposure UVC dose until a threshold UV dose (*Hs*).

To model the phenomenon, the foundations of new models were proposed associating the

growth kinetic of biofilm and the UVC dose effect on biofilm production:

$$(dM_b/dH_S) = \mu . dM_a/dH_S \quad (1)$$

Where; M_b: The total biomass of the biofilm continues to grow for the control test (before exposure to UVC light; H_{UV}=0 mJ.cm^{-2}); M_a: Active bacteria responsible for production and maturation of biofilm; μ: Biofilm growth rate; and H_S: threshold UV dose(mJ.cm^{-2}).

The values of threshold UV doses were different in relation with the response of each bacteria strain to UVC dose (dose/response). For example, for *P. aeruginosa* ATCC 15422 (PA_{ref}), the biofilm production is induced until a threshold UVC dose equal to 30 mJ.cm^{-2}. The threshold UVC dose is equal to 10 and 40 mJ.cm^{-2} for tested bacteria PA_1 and PA_3 respectively and it"s equal to 20 mJ.cm^{-2} for, *P. aeruginosa* strains PA_2 and PA_5.

The intra-specific difference showed in the UV dose/response relationship is probably dependent on several factors: the degree of DNA damage induced by UV, the speed of induction of DNA repair mechanisms for each tested bacteria. In fact, after UV irradiation, to limit UV damages, bacteria generally possess molecular mechanisms to restore DNA lesions. Bacteria have developed different repair pathways, including photoenzymatic repair (PER), nucleotide excision repair (NER) also called dark repair, and recombinational repair (post replication repair). PER involves direct monomerization process which is termed photoreactivation of CPDs by a single enzyme (photolyase) with near-UV or visible light as a source of energy (Kim *et al*., 2001). In contrast to photoreactivation, the mechanisms of dark repair are most complex repair process that involves the coordination of numerous enzymes to remove DNA damage.

Many studies were elucidating the central role played by the *recA* product, RecA protein, in the inducing and the coordination of different repair mechanisms. Indeed, RecA is a protein with a central role in DNA stability and repair under stress conditions leading to DNA damage (Kowalczykowski et al., 1994). RecA has multiple functions affecting different cellular processes, such as genetic recombination, the rescue of stalled or collapsed replication forks „Miller and Kokjohn (1988), and the replication of damaged DNA through translesion DNA synthesis (TLS) by DNA polymerase V (pol V) (Schlacher et al., 2006). Moreover, it is a key component for LexA self-cleavage. These processes require the interaction of filament. In addition a novel role for RecA was divulged consistant of the promotion of swarming motility in *E. coli* K-12 (Gómez-Gómez et al., 2007).

The increase of biofilm production can be related by the induction of mechanisms of repair of damaged DNA bacteria. Recently, Inagaki et al. (2009) showed that RecA with s ingle-stranded (ss) DNA forming a helical nucleoprotein RecA-deficient mutant strain (RAD) of *Streptococcus pneumoniae* showed reduced acid tolerance and produced lower density biofilm compared with the wild-type strain. In addition, confocal microscopic observation indicated that the biofilm produced by RAD was composed of cells with significantly lower viability compared with that produced by wild-type strain. These results suggest that RecA has a relationship with biofilm formation.

For consequence we can conclude that UVC irradiation by a moderate dose located between 10 and 40 mJ.cm^{-2} determined by this study induces *recA* gene and for consequence, increase the intracellular content of a multi-functional RecA protein causing the induction of DNA repair systems and the stimulation of biofilm production as a protective structure against UV irradiation. Indeed, Miller et al. (1999) was claimed that the alginate biofilm matrices transmits only a small amount of UV radiation (13% of UVC, 31% of UVB, and 33% of UVA), thus protecting the cells from exposure and suggesting that the exopolymer may be a natural defense mechanism used to attenuate UV light exposure in nature.

In addition, another study provided evidence that *P. aeruginosa* undergoes rapid genetic diversification during growth in biofilm communities (Boles et al., 2004). The genetic changes arise via a RecA-dependent mechanism, which likely involves recombination functions and affects multiple biofilm traits.

After irradiation of bacteria by UVC dose over than threshold dose (*Hs*), we were noted a progressive decrease in the production of biofilm correlated with the increase of UV dose. We can model this fact by the occurring equation:

$$(dM_b/dH_t) = \mu . M_a . [1- (dM_d/dH_t)] \quad (2)$$

Where; M_b: The total biomass of the biofilm continues to grow for the control test (before exposure to UVC light; H_{UV}=0 mJ.cm^{-2}); M_a: Active bacteria responsible for production and maturation of biofilm; M_d: Inactive bacteria no longer involved in the construction of biofilm; μ: Biofilm growth rate and; H_t: UVC dose over than *Hs* dose (mJ.cm^{-2}).

This decrease of biofilm production can be explained by the fact that the bacterial strains have received a lethal UV dose reducing bacterial sustainability by accumulation of photoproduits surpassing the capability of bacteria DNA repair mechanisms allowing for consequent, a decrease of biofilm formation and the weakening of this resistant structure. In addition, in bacteria, the regulation of many important changes in gene expression is mediated by systems of signaling between cells known as QS cells (Girard and Bloemberg, 2008). Cells in a population will sense their density and number through the presence of signals that diffuse freely across cell

membranes and between cells. Via an auto-induced positive feedback mechanism, a population of cells can quickly induce the appropriate phenotypes required for responding to a particular environmental condition or for proceeding with the differentiation process of the population (Kjelleberg et al., 2002; Parsek and Greenberg, 2000). The diverse small signal molecules used for quorum sensing (QS) transform independent cells into specialized cell communities. These small molecules are used to regulate biofilm formation (Ueda et al., 2009). *P. aeruginosa* possesses two quorum-sensing systems, *las* and *rhl* (Shrout et al., 2006). Each system has its own signal synthase, signal receptor and distinct acylhomoserine lactone (AHL) signal. Quorum sensingregulated functions are known to be critical for acute virulence (Shrout et al., 2006).The *Pseudomonas* quinolone signal (PQS, 2-heptyl-3-hydroxy-4-quinolone) also controls the production of pyocyanin, rhamnolipids and elastase (Diggle et al., 2006). The hierarchy of QS regulation is that the LasRI controls RhlRI regulation, and the PQS system is related to both the LasRI and RhlRI systems (Diggle et al., 2006). Consequently, these QS systems regulate virulence factors such as extracellular enzymes (LasA protease, LasB elastase and alkaline proteases), metabolites (pyocyanin and hydrogen cyanide), and biofilm formation that cause persistent infections by *P. aeruginosa* (Hammer and Bassler, 2003).

Knowing that QS defined as cell-density-dependent control of gene expression, the UVC irradiation of tested strains by dose inferior or equal to *Hs*, not inhibit the cell communication and allowed the formation of biofilm as bio-protection structure against germicide UV light. In contrast, the irradiation of *P. aeruginosa* strains by UVC dose over than the threshold dose (H_t), have an inhibitor effect on the cell-to-cell communication via diffusible signal molecules due to the decrease of active bacteria density (M_a) and the increase of defectives ones (M_d) (Equation 2).

Noted that, biofilm production was not inhibited after irradiation of *P. aeruginosa* strain PA_3, by a recommended dose for water disinfecting water equal to 40 mJ.cm^{-2} (Figure 1). This result, suggested that this dose is insufficient to inhibit the cell-to-cell communication system. Consequently, this applied UV dose for water disinfection does not able to repress the eventual expression and production of virulent factors by *P. aeruginosa* strain.

The retention of virulence factors after UVC disinfection make as thought to introduce new recommendation to valid UV disinfection system. The UVC required dose must be determined based on the repression of virulent factors expressed by model bacteria or indicator bacteria such as production of biofilm, bacterial motilities, proteases activity, etc.

P. aeruginosa has proved to be a biological model very interesting for the (i) estimation of virulence after water treatment, and (ii) the determination of the lethal dose allowing bacterial inactivation and attenuation of pathogens characters.

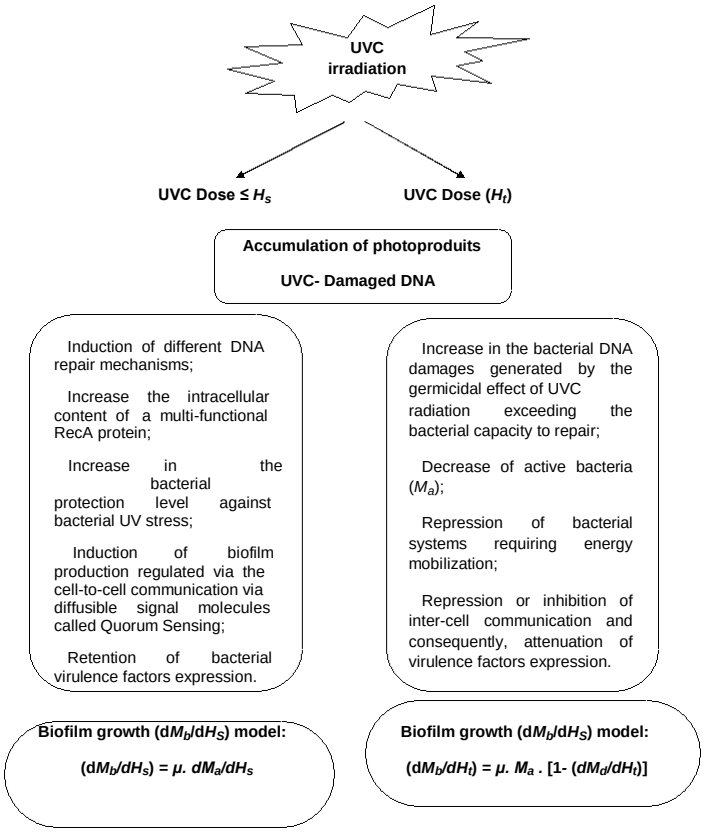

Figure 2. UVC Dose effet on biofilm production.
Where; M_b: The total biomass of the biofilm continues to grow for the control test (before exposure to UVC light; H_{UV}=0 mJ.cm^{-2}); M_a: Active bacteria responsible for production and maturation of biofilm; M_d: Inactive bacteria no longer involved in the construction of biofilm; μ :Biofilm growth rate; H_s: threshold UV dose(mJ.cm^{-2}) and H_t: UVC dose over than *Hs* dose (mJ.cm^{-2}).

In the end of this study, we are established a probably link between UVC dose – Inducible DNA repair and damage control system – regulation of virulent factors (QS) (Figure 2). In addition we are introducing the perspective study interesting for the determination of UV disinfection system performances based on exploration of bacterial virulence factors.

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