Inactivation of a wild isolated *Klebsiella pneumoniae* by photo-chemical processes: UV-C, UV-C/H_2O_2 and UV-C/H_2O_2/Fe^{3+}

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Abstract

Shrimp (*Litopenaeus vannamei*) farming is an important economic activity in several countries . Pathogens in shrimp farms and its effluents pose a potential hazard for both humans and shrimps. Wild strains of bacteria were characterized in a shrimp farm, and *Klebsiella pneumoniae* was chosen as a good indicator due to its presence in the pond and the effluent and its resistance to antibiotics. Different photochemical processes (UV/H_2O_2, $UV/H_2O_2/Fe^{3+}$) were tested for inactivation of wild isolated *K. pneumoniae* and compared to UV-C radiation. By kinetic modelling, a k_{max} equal to 0.43 s^{-1} was obtained for UV-C treatment. After optimizing the cited processes, ranging [H_2O_2]: 10-30 $mg \cdot l^{-1}$; an optimal [H_2O_2] of 10 $mg \cdot l^{-1}$ was found, increasing k_{max} on 13.63% compared to UV-C. This optimal concentration was tested for $UV/H_2O_2/Fe^{3+}$ process; ranging [Fe^{3+}]: 2-20 $mg \cdot l^{-1}$. The highest yield was obtained by a [H_2O_2]:[Fe^{3+}] = 10:2, which leads to 4-Log reduction in 12.88 s of treatment. Moreover, resistance of *K. pneumoniae* was compared to *Escherichia coli*. The latter proved to be more sensitive despite its similar cellular structure. Results suggested that the photochemical processes could enhance disinfection efficiency, especially for photo-assisted Fenton-like process in most resistant bacteria.

Keywords

Aquaculture; Iron; Antibiotic resistance bacteria; Fenton process; Advanced oxidation processes; Hydrogen peroxide.

1 Introduction

Globally, aquaculture accounts for almost 50% of fish destined for food and is the fastest growing food sector [1]. More than 550 aquatic species are currently grown around the world. Within these species, shrimp (*Litopenaeus vannamei*) and its cultivation is an important economic activity in several countries, such as China [2], Thailand [3], Vietman [3], India [4,5], Brazil [6] and Costa Rica [7] is one of the most important economic activities in Ecuador [1].

Ecuadorian shrimp exports increased from $1,278 to $2,580 million dollars between 2012 and 2016, when they were the third most exported product, representing 15% of the country's exports [8]. With an exponential growth, shrimp farms amounted to about 210,000 hectares in 2016 along the four coastal provinces of Ecuador (El Oro, Guayas, Manabí and Esmeraldas). In the province of El Oro, in southern Ecuador, shrimp farming has contributed to improving the socio-economic situation of the region. However, shrimp farms take up large areas of mangrove, estuaries and coastal bays and provoke a remarkable environmental impact in areas with great environmental value.

The high concentrations of shrimp, fecal matter and unconsumed organic fertilizers favor the growth of pathogenic microorganisms, which are an important source of disease and mortality in shrimps, causing economic losses [9]. Those pathogens are normally prevented by the extensive use of antimicrobials, leading to the presence of multiple resistant bacteria in the cultured shrimps, including cited pathogens, making the control of them extremely challenging [10]. Effluents from this activity are released into the surrounding aquatic environment and, as a consequence of that, aquaculture activities appears among one of the main sources of Antibiotic Resistance Bacteria (ARB) in the environment, considered as contaminants of emerging concern [11], posing a potential ecosystem deterioration and health hazard [12,13].

Thus, water treatment technologies, especially for disinfection purposes, are needed in order to assure water quality for increasing process efficiency and generating safe discharges to natural environment. Several disinfection techniques are available, like chemical disinfectants and antibiotics. However, these methods proved to be insufficient against resistant pathogens not guaranteeing complete disinfection [14]. Sodium hypochlorite (NaClO) is a common disinfection chemical because of its low cost and high effectiveness.

Nevertheless, chlorination could generate potentially harmful chloro-organic by-products when natural organic matter is present [15].

Ultraviolet (UV) irradiation is a well-established treatment that disinfects without by-products due to absorption of UV by organic molecules, such as DNA [16–18]. However, microorganisms are capable of repairing themselves [19,20]. UV irradiation efficiency can be enhanced by generating radicals (mostly hydroxyl radical, •OH) with several catalysts (TiO_2, Iron) or oxidants (H_2O_2, O_3) that can be photo-activated. •OH is a powerful oxidizing agent with a short lifetime that lacks the potential for environmental damage [21]. Processes in which •OH is involved are called Advanced Oxidation Processes (AOPs) [22,23] and are capable of inactivating microorganisms by degradation of the chemical structure of cell walls [24].

Some of these UV-based technologies have been studied as alternatives to marine water disinfection treatment: UV/TiO_2 [25,26], UV/O_3 [27,28], etc. However, TiO_2 requires catalyst cleaning [25] and O_3 generates by-products [17]. Thus, the use of H_2O_2 appears to be a promising alternative.

The photolysis of H_2O_2 generates •OH following Eq. (1) [22]. Iron, in conjunction with H_2O_2 and light, acts as a catalyst and increases the production of •OH in an AOP known as photo-assisted Fenton process. This is a cyclic catalytic process that generates •OH transforming Fe^{3+} to Fe^{2+} and vice versa. Depending on iron source, it is so-called Fenton-like reaction (Fe^{3+}). Among different AOPs, the photo-assisted Fenton process has attracted great attention due to its high generation of •OH. The process can be summarized in Eq. (2) and (3) [15,29].

$$H_2O_2 + hv \rightarrow 2\ \bullet OH \qquad \text{Eq. (1)}$$

$$Fe^{3+} + H_2O + hv \rightarrow Fe^{2+} + \bullet OH + OH^- \qquad \text{Eq. (2)}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^- \qquad \text{Eq. (3)}$$

UV/H_2O_2 and photo-Fenton ($UV/H_2O_2/Fe^{2+,\ 3+}$) processes are both effective at disinfection in both natural water and wastewater [30–32]. Even though there were doubts of the effectiveness of these treatments for seawater disinfection because of the high concentration of inorganic ions, which can interfere by •OH scavenging in AOP applications [22,33,34], some authors have already demonstrated effectiveness [15,35,36].

In this context, the main objective of this work is to identify and isolate pathogenic microorganisms present in effluents from shrimp farms located in southern Ecuador and evaluate the inactivation efficiency of different processes: UV-C, UV-C/H_2O_2 and UV-C/H_2O_2/Fe^{3+} by using two different strains: a wild isolated bacterium from a shrimp farm (*Klebsiella pneumoniae*) and a typical microbial indicator (*Escherichia coli*).

2 Materials and methods

2.1 Water sampling

In order to identify and isolate different microorganisms present in shrimp farm effluents, water samples were collected from a shrimp farm located in Huaquillas canton (3°28′52.97″ S 80°14′36″ W), El Oro province, southwestern Ecuador. The climate of this area is warm and dry with a temperature of 20 to 35ºC throughout the year.

Three sampling points were selected in the shrimp farm (influent, pool and effluent). Each water sample was collected in 20 liter plastic drums. The samples were transported at 4°C and in the dark to the laboratory of the Department of Chemistry and Exact Sciences of the Universidad Técnica Particular de Loja. Additionally, water characterization (microbiological and physico-chemical) was performed in each sampling point (Table S1. Suppl. data).

2.2 Bacterial isolation and identification

2.2.1 Bacterial isolation

In order to isolate different microorganisms, water samples were filtered twice. Firstly, three liters of sample were filtered with Whatman No. 1 filter paper to remove plant residues and sediments. Secondly, they were filtered on a 0.45 µm membrane filter (Neogen Filter). Five milliliters of alkaline peptone (APA Merck KGaA, pH 8.5) culture medium were added to the membrane-containing residue and incubated at 37°C for 24 hours. Samples were cryogenically preserved (-75ºC) after resuspending in 900 µl of the culture medium with 100 µl of dimethylsulfoxide (DMSO 10%).

Isolation and purification of microorganisms were carried out from the cryogenic reserves following the quadrant streak technique over a series of commercial media: APA agar, blood agar, MacConkey agar, TCBS and GSP agar. Finally, the plates were incubated at 37°C for 24 hours. Colonies with good morphology were extracted and spread over the same media

where initially isolated. The purity of the cultures was reviewed by macroscopic analysis on agar plates (shape and color of colony) and microscopic examination of the developed colonies (wet amount and Gram staining) [37]. Additionally, the occurrence of some commonly used biochemical markers was identified by means of oxidase (cytochrome c oxidase), catalase (catalase enzyme produced by organisms that live in oxigenated environments) and indole (tryptophanase system) tests. The oxidase test is a key test to differentiate between the families of Pseudomonadaceae (ox +) and Enterobacteriaceae (ox -) [38] .

2.2.2 Bacterial identification

Molecular identification was carried out through DNA extraction (Pure LinkTM Genomic DNA mini Kit) by the protocol for Gram-negative or Gram-positive bacteria, according to the manufacturer's specifications. The partial 16S rDNA was amplified using universal primers 27F 5'-AGAGTTTGATCMTGGCTCAG-3' [39] and 1492R 5'-GGTTACCTTGTTACGACTT-3' [40]. The PCR products were purified with the Wizard® SV gel and PCR Clean-Up System kit, and the presence of amplicons was verified by GelRed® stained (Biotium, Hayward, CA, USA) in 1% agarose electrophoresis gels. All the purified products were sequenced by Macrogen (Seoul-Korea).

The sequences obtained were compared to the closest reference sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). In addition, our sequences and the closest sequences from the database were aligned using the MAFFT software (G-INS-I strategy) in order to build the phylogenetic trees for each group of bacteria. The phylogeny was generated by Maximum Likelihood (ML) analysis implemented in the MEGA v5 software.

2.3 Inactivation assays

2.3.1 Microbiological procedures

Among the microorganisms detected and isolated, *Klebsiella pneumoniae* cultures were chosen as indicator bacteria. First, from a cryogenic reserve, an aliquot of 0.1 ml was taken to incubate over Tryptic soy broth (BD soybean broth, pH 7.3±0.2) at 37°C for 12-14 hours. After this, the inoculum was adjusted to 0.5 McFarland with a densitometer (Grant instrument, DEN-1) and, finally, 1 liter of water matrix was inoculated with the culture in a ratio 1:10 to adjust to a final concentration of ca. 10^7 CFU·ml^{-1}.

Escherichia coli (ATCC 25922®) was used as control strain for its prevalence in waste water. It was seeded over Violet Red Bile Agar (BD-Difco) and inactivated under the same conditions given above for *K. pneumoniae* to compare the effectiveness of the different treatments.

After inactivation assays, 10 ml aliquots were transferred into sterile tubes from the reactor outlet and seeded over trypticase soy agar (BD-Difco, pH 7.3±0.2). The number of colony forming units per milliliter ($CFU \cdot ml^{-1}$) was quantified by colony counting. To do this, 100 µl of each aliquot from every treatment were spread with a sterile L-shaped Drigalski loop until the medium absorbed it completely. Petri plates were inverted and incubated at 37°C for 24 hours. This procedure was performed in duplicate and only measurements with a coefficient of variation fewer than 30% were considered. In case the count was very high, appropriate dilutions were made until obtaining an optimum count (10-100 CFU). The bacterial suspension was quantified before adding to the reactor, by the same technique, to assure an inoculum of 10^7 $CFU \cdot ml^{-1}$. Control samples were performed in each assay to assure that bacterial concentration did not change during experimental procedures.

2.3.2 Experimental assays

The inactivation experiments were carried out using a continuous reactor consisting of a peristaltic pump feeding system (Cole-Parmer Master Flex L/S Digital Drive Model 7523-80) with a central UV-C lamp (6W – Low Pressure Hg) in a tube of quartz and stainless steel with a plastic shell (Ultraviolet Sterilization Filter, Model: OPP-625, Microfilter Co. Ltd.). Irradiated volume was 140 ml with a mean intensity of 16.7 $\mu W/cm^2$.

Three treatments were performed: UV, UV/H_2O_2 and UV/H_2O_2/Fe^{3+}. Chemicals used were H_2O_2 (30% by weight, Merck) and Fe^{3+} (Iron(III) chloride hexahydrate, Merck, ACS). In order to optimize different processes, different concentrations of H_2O_2 and Fe^{3+} were evaluated according to Table 1. Water matrix for experimentation was prepared by dissolving NaCl (Merck, ACS) at 35 $g \cdot l^{-1}$ Milli-Q water. H2O2 and Fe were measured in some assays before and after the treatment with peroxide tests for H2O2 (colorimetric test strips method, 0.5–25 and 1–100 mg/L H2O2 Merckoquant–Merck) and flame atomic absorption spectrometry (Perkin Elmer AAnalyst 400) for Fe, finding no consumption

Table 1. Experimental design for evaluation of different inactivation processes for *E. coli* and *K. pneumonia*.

Treatment	H_2O_2 (mg·l^{-1})	Fe^{3+} (mg·l^{-1})	Retention time (s)
UV	0	0	16, 26, 36, 46, 56
UV/H_2O_2	10	0	
	30	0	
$UV/H_2O_2/Fe^{3+}$	10	2	
	30	6	

2.4 Data treatment

Inactivation of bacteria is represented as the logarithm of the ratio between the number of bacteria after a time t of exposure in the reactor and the number of initial bacteria. The GinaFiT software, a plug-in for Microsoft Excel, was used to find which mathematical model best fits the data obtained and to calculate inactivation kinetics, k_{max} (s^{-1}) [41]. Only those kinetics which explanatory mathematical model obtained a coefficient of determination (R^2) greater than 0.9 were accepted.

SPSS version 20 was used to carry out a normality test (Shapiro-Wilk) as a preliminary step to ANOVA analysis to verify whether there were significant differences in bacteria inactivation between the treatments used (UV, UV/H_2O_2, $UV/H_2O_2/Fe^{3+}$) according to the experimental design shown in Table 1.

3 Results and discussion

3.1 Bacterial identification

Four microorganisms were initially purified over different culture media. Morphological analysis of bacteria was used only to determine the purity of the cultures but molecular analysis was crucial to identify the species.

Molecular analysis showed four species, confirming Gram-negative and Gram-positive bacteria (Table 2). Only two strains were defined to species level, and two were defined to genus level due to the lack of species information in the GenBank database. The BLAST sequence identifications were supported by maximum likelihood in the phylogenetic trees for each genus (trees not shown) with bootstrap values >80% by clade.

196 **Table 2.** Bacterial identification in water samples from the shrimp farm. Percentages of
197 similarity are between our new sequences and the sequences available from the GenBank
198 database.

199

Strains code	Identification		Similarity	Query cover	Accession number
	Gram-definition	**BLAST**			
PAMR	Gram-negative	*Klebsiella pneumoniae*	99%	100%	LC216325
FPAC	Gram-negative	*Vibrio fluvialis*	99%	100%	KT163389
PAAS	Gram-positive	*Bacillus* sp.	99%	100%	KJ473716
FPAN	Gram-positive	*Exiguobacterium* sp.	100%	100%	KX911472

200 *K. pneumoniae* was isolated after growing on MacConkey agar as a mucoid, convex and
201 lactose positive colony, and it showed the characteristics of a Gram-negative bacteria after
202 Gram staining and was indole negative, oxidase negative, catalase positive. *K. pneumoniae*
203 was isolated from the water collected inside the pond and in the effluent of the pond.

204 *Vibrio fluvialis* was isolated after growing on TCBS agar as medium-sized, smooth, yellow
205 colonies and displayed the characteristics of a Gram-negative, motile bacterium. It was
206 indole negative, oxidase positive. *V. fluvialis* was isolated from the influent of the pond.

207 *Bacillus* sp and *Exiguobacterium* sp. were isolated over Alkaline Peptone agar (APA
208 medium) as small convex colonies, *Exiguobacterium* sp. revealed a characteristic orange
209 appearance on the plate and it was a rod-shaped Gram-positive bacteria. *Bacillus* sp.
210 exhibited an opaque appearance in pale yellow and dry colonies, and it was a rod-shaped
211 Gram-positive bacterium after gram staining. *Bacillus* sp was isolated from the water inside
212 the pond whereas *Exiguobacterium* sp. was isolated from the influent of the pond.

213 The presence of microorganisms in the influent, such as *V. fluvialis*, *Bacillus* sp and
214 *Exiguobacterium* sp., could be explained by wastewater discharges in the area from the city
215 of Huaquillas. In this case, *K. pneumoniae* would also be expected to be present.

216 In contrast to the other strains identified, *K. pneumoniae* was the only one detected in both
217 pond and effluent samples, suggesting that this bacterium is more resistant to the antibiotics
218 used by the farmers than the rest of the microorganisms identified. *K. pneumoniae* is
219 considered a problematic pathogen and could be a significant risk in both human health and
220 shrimp production [32,42]. Furthermore, it has special defense mechanisms against different
221 bactericides and antibiotics [43,44]. Thus, AOPs are especially interesting for *K.*

pneumoniae inactivation in shrimp farms. Further research will be carried out to inactivate the rest of the isolated bacteria.

3.2 Inactivation assays

In order to evaluate the disinfection efficiency of several UV-based processes for treatment of aquaculture effluents, different inactivation assays were performed with wild bacteria isolated in Section 3.1 (*K. pneumoniae)* as a microorganism indicator. Results were compared with a typical indicator, *Escherichia coli* (ATCC 25922®), as shown in Figure 1. Plate counting afforded a detection limit of 2 CFU·ml^{-1}.

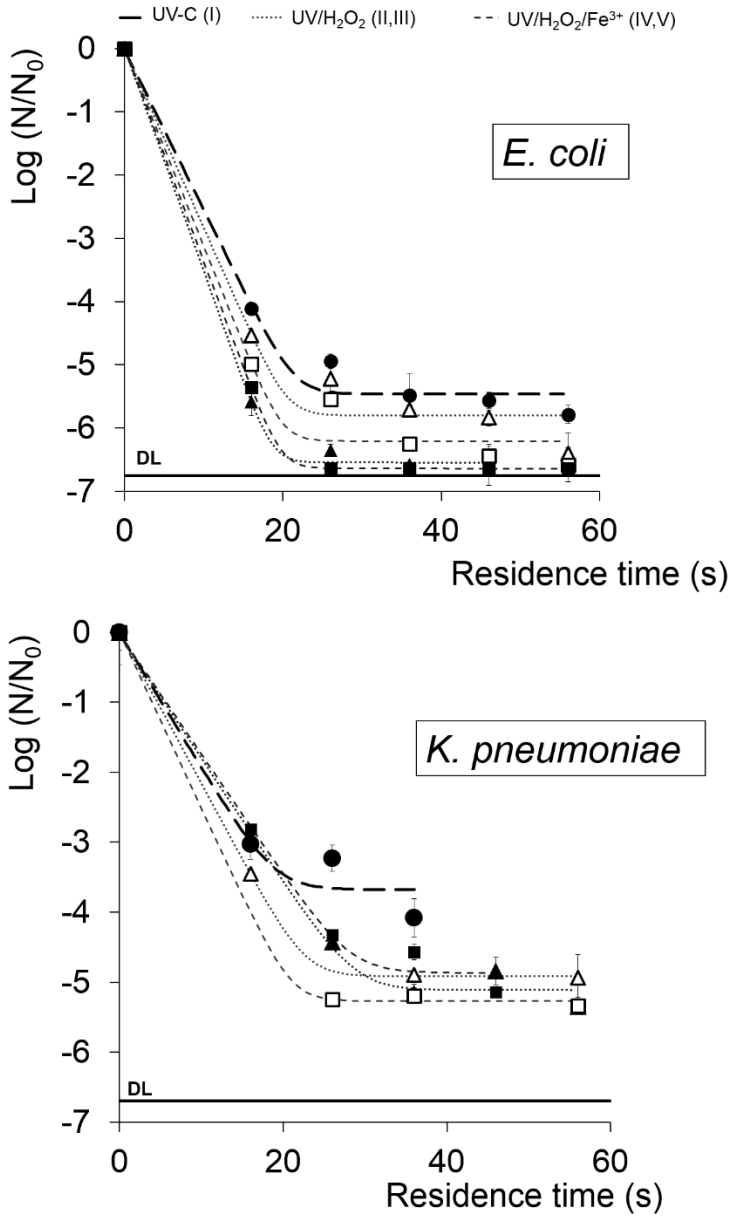

Figure 1. Inactivation profiles of *Escherichia coli* and *Klebsiella pneumoniae* under several disinfection processes. Symbols represent the average of experimental points and lines show

a fit by Log-lineal + tail model: (I) ●UV-C; (II) △ UV/H_2O_2, [H_2O_2]=10 mg·l^{-1}; (III) ▲ UV/H_2O_2, [H_2O_2]=30 mg·l^{-1}; (IV) □ UV/H_2O_2/Fe^{3+}, [H_2O_2]=10 mg·l^{-1}, [Fe^{3+}]=2 mg·l^{-1}; (V) ■ UV/H_2O_2/Fe^{3+}, [H_2O_2]=30 mg·l^{-1}, [Fe^{3+}]=6 mg·l^{-1} DL: Detection Limit

Inactivation of bacteria is shown in Figure 1 as logarithmic reduction of the surviving bacteria vs. retention times in the UV-reactor. Experimental raw data were fitted into Log-Lineal + tail model (R^2 >0.9), according to Eq. (4) [45].

$$N_t = (N_0 - N_{res}) \cdot e^{-k_{max} \cdot t} + N_{res} \quad \text{Eq. (4)}$$

Where N represents the concentration of viable bacteria, in CFU·ml^{-1}, at a given time (N_t) and before treatment inactivation (N_0); fraction related to the tailing phenomenon (N_{res}); k_{max} is the inactivation rate of the Log-lineal + tail model (s^{-1}) and "t" is the retention time (s) in the UV-reactor.

According to the results obtained, *K. pneumoniae* was observed to be more resistant than *E. coli*. For *E. coli*, 4 log-reductions (99.99%) were reached in only 16 s of exposure to all treatments studied: UV-C (I), UV/H_2O_2 (II, III) and UV/H_2O_2/Fe^{3+} (IV, V). In the case of *K. pneumoniae*, higher retention times were needed to reach 4 log-reductions: UV/H_2O_2 and UV/H_2O_2/Fe^{3+} achieved it with 26 s of exposure to UV light. The treatment exclusively with UV light required 36 s to achieve the same degree of inactivation.

A deeper analysis, based on k_{max} (s^{-1}) was performed for different processes applied in order to detect significant differences in inactivation kinetics between *K. pneumoniae* and *E. coli*. Results are shown in Figure 2.

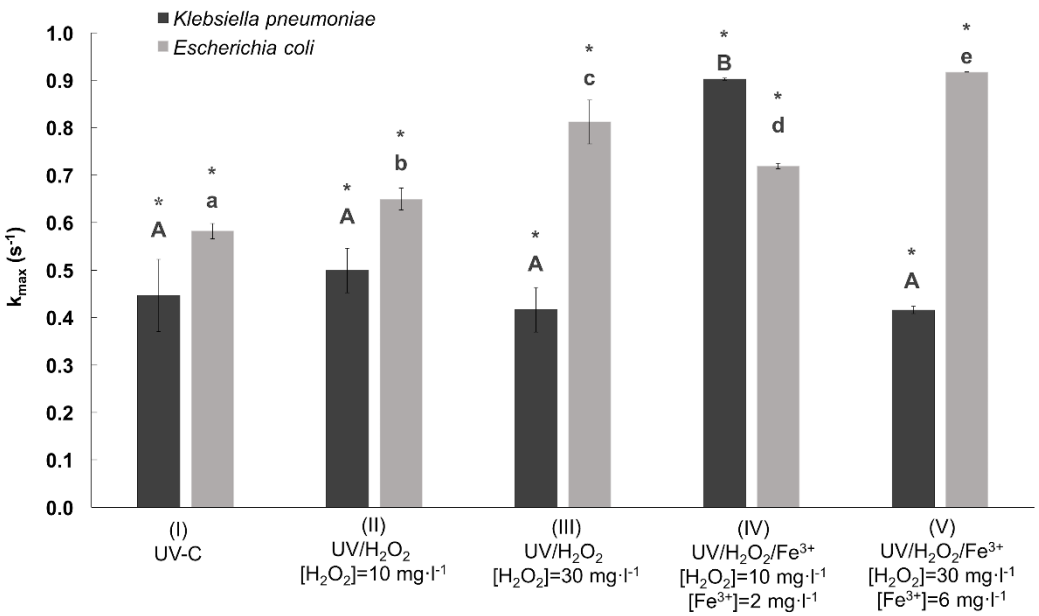

Figure 2. Kinetic rate constant, k_{max} (s^{-1}), obtained in different disinfection processes for *Klebsiella pneumoniae* and *Escherichia coli*. Error bars depict the standard deviation. Asterisks (*) show data for differences ($p<0.05$) between bacteria (*K. pneumoniae* - *E. coli*) in the same treatment. Capital letters show significant differences ($p<0.05$) between treatments for *K. pneumoniae* (A–B) and lowercase for *E. coli* (a–e). I.e. treatments with the same letter have no significant differences among them, compared to treatments with different letters, between which there are significant differences.

It was detected that the inactivation was always greater for *E. coli*, suggesting a major sensitivity of this bacterium compared to *K. pneumoniae*. The origin of the strain could be relevant; while *E. coli* is a commercial strain, *K. pneumoniae* has been isolated from the wild. Also, cell wall structure has a significant role in UV-light processes: when UV is applied, genetic material and proteins are the main biomolecules affected, principally because of the high absorption caused by pyrimidine bases [46]. Sensitivity also depends on cell wall characteristics (Gram-positive or –negative) [47,48]; however, *E. coli* and *K. pneumoniae* are both Gram-negative bacteria, with the same cell wall structure.

E. coli shows high sensitivity to UV light alone; e.g., some authors, [25,49], reached rapid inactivation of this organism at short exposure times. Others, [50], reported more resistant properties for *K. pneumoniae* than *E. coli*. Hijnen et al. [16] compared the resistance of different microorganisms, including bacteria and bacterial spores. Their study suggested an increased UV-resistance for wild strain bacteria. Apart from the nature of the strain, it was

reported that *K. pneumoniae* has a special mechanism against bactericidal processes, such as large cell aggregations or production of extracellular polymers [43,44].

When H_2O_2 is added to the UV-C process, the photolysis of H_2O_2 generates •OH which enhance the disinfection efficiency. Nevertheless, H_2O_2 overload brings a recombination phenomenon and consequent scavenging of •OH, reducing disinfection yield [51]. To avoid H_2O_2 in excess, two different concentrations were tested (II, III). Previously, several concentrations over 30 $mg \cdot l^{-1}$ were tested but no significant differences in disinfection were found. In this sense, inactivation efficiency of *E. coli* improved ($p<0.05$) by adding H_2O_2 to a water matrix, showing a better yield at the higher concentration of H_2O_2 (III, 30 $mg \cdot l^{-1}$) than at the lower (II, 10 $mg \cdot l^{-1}$). It is probably due to the major reaction capacity of the generated •OH which has a strong effect on cell inactivation.

Nonetheless, the same effect was not observed for *K. pneumoniae*. Since the kinetic rate constant is higher by adding H_2O_2 (in comparison with UV alone), no significant differences were detected with 10 and 30 $mg \cdot l^{-1}$ of H_2O_2 (II, III). As previously noted, *K. pneumoniae* has a robust capsule that can protect it from direct attacks from oxidant radicals. *E. coli*, however, showed high sensitivity to UV light alone, especially at high intensities [52,53]; it could enhance the inactivation caused by the generation of •OH [25,49]. Moreover, the slight effect that H_2O_2 might cause by diffusion mechanisms into the cell could generate an extra-effect due to the intracellular iron and could explain those differences[14,15,32,54]. Those results are in agreement with previous studies [49].

By adding Fe^{3+}, the inactivation yield improved (*E. coli*) compared to treatment I, II and III; mainly at high concentrations (V). We could assume, as explained previously, that the enhancement of disinfection yield could be due to the UV-C sensitivity of this bacterium, accentuated by the presence of oxidants, rather than the impact of •OH itself [25,49].

For *K. pneumoniae*, results differ as with I, II, and III processes. The inactivation rate is improved again by adding Fe^{3+}, although the cited improvement is only significant ($p<0.05$) for treatment IV which has been the most efficient treatment and the only treatment that showed significant differences with the rest.

In the photo-assisted Fenton-like process, different concentrations have been tested: 10, 30 $mg \cdot l^{-1}$ H_2O_2 together with 2, 6 $mg \cdot l^{-1}$ of Fe^{3+}, respectively. Disinfection efficiency improved significantly when low concentrations were applied (IV), i.e., the effect of the iron catalyst is enhanced at 10 $mg \cdot l^{-1}$ of H_2O_2 instead 30 $mg \cdot l^{-1}$. It can corroborate the optimum

concentration of 10 mg·l^{-1} of H_2O_2 for both UV/H_2O_2 and UV/H_2O_2/Fe^{3+} processes within the concentrations tested because of optimal radical generation and avoidance of recombination processes. As a result, an iron-catalyzed process on *K. pneumoniae* has a significant effect on cell inactivation

It is known that ferric salts catalyze the generation of •OH in the so-called Fenton-like reaction. In this case, radicals are generated by reduction of Fe^{3+} to Fe^{2+}, mainly by H_2O_2 and light-assisted processes [55,56]. Even though many studies reflect the major efficiency of Fe^{2+} as the starting iron species compared to Fe^{3+} [32], the natural form of iron is mostly as Fe^{3+} which encourages assessing Fe^{3+} efficiency and its potential as a source of iron. . On the same way, it has to be taken into account that this process with Fe^{3+} is limited by pH, which tends to precipitate at near neutral values. Moreover, since the Fenton-like process seems to be light-dependent (unlike the Fenton process) [57], the use of UV-C as a source of light could enhance the disinfection efficiency. Despite these assumptions, a disinfection mechanism based on cell adsorption could explain the enhancement of the process: Fe^{3+} has high charge density, and it can be attracted by bacterial cells; so the oxidizing radical's formation close to the cells may cause membrane damage which, together with the H_2O_2 in solution that increases permeability of the membrane (because of diffusive processes), could be the reasons for those disinfection improvements [57]. Spuhler et al. [57], demonstrated that most Fe^{3+} was retained together with the bacterial pellets when it was filtered, indicating that Fe^{3+}, in some circumstances, could be more effective than Fe^{2+} in solution.

In this study, the use of Fe^{3+} together with H_2O_2 and UV-C light significantly improved the inactivation. These results are in agreement with previous studies in which virus [58] or bacteria [44,57] are target organisms. In these studies, inactivation efficiency with Fe^{3+} and natural iron in solution was enhanced, compared to UV and/or UV/H_2O_2 processes..

Another important factor for AOPs application is the composition of the water matrix. In this study, a synthetic water matrix based on distilled water and NaCl in high concentrations similar to seawater (≈ 35 g·l^{-1}) has been used. Such chloride concentrations can interfere with disinfection processes by scavenging •OH and generating inorganic radicals (•Cl and •Cl_2) that could be less reactive [34]. The slight improvements with *K. pneumoniae* when H_2O_2 is added can be attributed to this scavenging effect of salts. However, those salinity effects seem to be small. For example, Penru et al. [33] achieved full disinfection in seawater; Moreno-Andres et al. [22] obtained efficiency losses of 5.22% compared to distilled water. Our results suggest a slight improvement (13.63 %) in kinetic rates for *E. coli* and *K.*

pneumoniae compared to UV-C. In real water matrices other ions and organic matter could be present in solution and interfere with disinfection processes. This is the case of Br^- and HCO_3^- in marine waters which have a strong scavenging rate that could lead in decrease disinfection efficiency [15,22,34]. Because of that, in further studies should be determined the weight of this scavenging and/or generation of other kind of radicals.

Fenton processes in water with high chloride content can be affected as well by formation of chloro-Fe^{3+} complexes ($FeCl^+$, $FeCl^{2+}$, $FeCl^{2+}$) which could decrease the generation of •OH and consequently lower the efficiency [59]. On the other hand, Spuhler et al. [57] reached high disinfection efficiencies, even in saline solution. Rubio et al. [15] significantly improved disinfection in artificial seawater under a photo-Fenton process. In our case, in the best situation (IV), an increase in kinetic rate constant (*K. pneumoniae*) of 50% has been achieved compared to UV-C and 44.4% compared to UV/H_2O_2.

4 Conclusions

Among different bacteria isolated in shrimp farms, *K. pneumoniae* was chosen as microbiological indicator for evaluation of different UV-based processes because it was the only bacteria identified in both the pond and the effluent. Compared to *E. coli* as typical microbial indicator, higher resistance was detected for *K. pneumoniae* in all processes tested. it is the best indicator for both AOPs evaluation of disinfection in this context and a good ARB indicator/model.

Considering inactivation kinetics, an optimal concentration of 10 $mg \cdot l^{-1}$ within the concentrations tested was obtained for the UV/H_2O_2 process; the same was obtained for UV/H_2O_2/Fe^{3+}, which was the most efficient treatment for the inactivation of *K. pneumoniae* among those tested. Those processes can improve the disinfection efficiency (based on k_{max}) by 13.63% and 50% respectively, compared to UV-C radiation. The increase in H_2O_2/Fe^{3+} concentrations did not always result in an increase in efficiency.

According to the results obtained, despite the possible effects caused by chlorides; the application of AOPs could enhance disinfection efficiency, especially for photo-assisted Fenton-like process in most resistant bacteria.

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