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*Research Paper*

# Effect of disinfectants on adhered *Aeromonas hydrophila* to polyethylene immersed in water under static and dynamic conditions

**Lontsi Djimeli C., *Nola M., Tamsa Arfao A., Nandjou Nguéfack R.V., Noah Ewoti O.V., Nougang M.E. and Moungang M.L.**
Laboratory of General Biology, Hydrobiology and Environment Research Unit, Faculty of Sciences, University of Yaoundé 1, P.O. Box 812, Yaoundé, Cameroon

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## Abstract

**The growth of *A. hydrophila* in liquid medium was monitored a hyperbola of four stages of growth. The effect of Sodium hypochlorite (NaOCl) (0.5, 1 and 1.5 ‰) and Hydrogen Peroxide ($H_2O_2$) (5, 10 and 15 ‰) on the adherent cells under static and dynamic conditions on fragments of polyethylene immersed in water was assessed. Cells were harvested from different growth stages. With cells harvested from each growth stage, abundances of adhered *A. hydrophila* were generally lower in the presence of NaOCl and $H_2O_2$ than in their absence. With the 2 disinfectants, a significant difference amongst the average densities of adhered *A. hydrophila* at each growth phase was observed ($P<0.05$). The effectiveness of each disinfectant concentration on adhered *A. hydrophila* decreased as the duration of the adhesion increased. Although the adsorption coefficient obtained from Freundlich isotherms was relatively higher in the static than in the dynamic regime, no significant difference was observed between the mean abundances of *A. hydrophila* adhered under these two experimental conditions ($P>0.05$). NaOCl seems more effective on *A. hydrophila* adhered to polyethylene than $H_2O_2$. Adhered *A. hydrophila* to polyethylene under the dynamic condition was more sensitive to both disinfectants than that adhered under static condition. These results suggested that the incubation duration and the cell growth stage played an important role in the bacterial resistance mechanism towards disinfectants.**

**Keywords:** adhered *A. hydrophila*, cell growth phase, effect, $H_2O_2$, NaOCl, water.

## Introduction

One of the major concerns of companies in charge of the treatment of drinking water is to effectively meet the demand and maintain the good quality of water in distribution [1]. The drinking water distribution network is often the place of many physico-chemical and biological reactions resulting in interactions between disinfectants, pipe walls, and free or fixed biomasses. These reactions are sometimes the cause of the deterioration of the organoleptic properties of supplied water [2, 3]. Analysis of the water distribution quality is based on physico-chemical and microbiological parameters [3, 1]. In recent years, the public health sector recognized *A. hydrophila* as an opportunistic pathogen, implicated in gastroenteritis, septicemia, cellulitis, colitis, meningitis and respiratory infections [4, 5, 6]. To prevent bacterial re-growth a residual disinfectant is maintained in the water distribution network. Ozone ($O_3$), chlorine dioxide ($ClO_2$), monochloramine ($NH_2Cl$), free chlorine ($Cl_2$), NaOCl, $H_2O_2$ are disinfectants that can be sometimes used in water disinfection treatment [7].

Previous works have shown that *A. hydrophila* is a widespread species in the environment. This microorganism has been isolated from lakes, rivers, sea water, sewage effluent, and especially in

water intended for human consumption [8, 9]. Its concentration is generally between 0 and $10^2$ CFU/ml in the outlet of drinking water treating plant. This concentration can be higher in drinking water distribution networks due to its growth on biofilms [10, 8]. Ingestion of contaminated food or water is the common route of advanced infection in the case of *Aeromonas* [11]. The pathogenicity of *A. hydrophila* is expressed primarily in fish, seafood and amphibians [12]. With the humans, *A. hydrophila* is an opportunistic pathogen that causes intestinal infections such as gastroenteritis [13] or extra-intestinal infections such as cutaneous infections [14]. They are responsible for gastroenteritis and severe diseases (septicemia, peritonitis ...), wound, genitourinary and ocular infections in immunodepressed patients [15]. One number of virulence factors contributing to its pathogenicity. Besides enzymes favoring infection (proteases, lipases, DNase), mobile *Aeromonas* have adhesion capabilities and produce various toxins including endotoxins, enterotoxins, hemolysins and cytotoxins [16].

Many studies have also focused on monitoring, water supply, treatment plants, and the health risks associated with the dysfunctioning of the distribution of drinking water [17, 18, 19]. They showed that despite treatment done upstream added to maintain a disinfectant residual in pipelines and oligotrophic medium, some bacteria adapt and proliferate in the water distribution network [1]. They are sometimes the cause of nests and microbial biofilm formation among others. In addition, the variation of microorganisms in response to disinfectants can be linked to changes in their cell wall which may be due to a change in their growth stage [20]. While previous studies have allowed to understand the mechanisms of emergence and evolution of biofilms of *A. hydrophila* in the drinking water distribution system [8, 1], there is little information on the importance of the growth stages or metabolic process and the reaction of biofilms of *A. hydrophila* against disinfectants. The present study aims at evaluating in microcosm the effect of NaOCl and $H_2O_2$ on *A. hydrophila* bonded under different conditions at different growth stages on polyethylene fragments immersed in water during time.

## Materials and Methods

### Collection and identification of *A. hydrophila*

The bacteria *A. hydrophila* was isolated from well water in Yaoundé (Cameroon) using membrane filtration technique, on ampicillin-dextrin agar medium [21, 22]. Cell subculture was performed on standard agar medium (Bio-Rad laboratories, France). The cells were then identified using standard biochemical methods [23]. These cells are anaerobic facultative, non-sporulated, Gram-negative bacilli, ferment mannitol, produce indole and mobile. They do not possess urease, lysine decarboxylase (LDC), ornithine decarboxylase (ODC) and arginine dihydrolase (ADH). For the preparation of stocks of bacteria, the Colony Forming Units (CFUs) from standard agar medium were inoculated into 100 ml of nutrient broth (Oxford) for 24 hours at 37 °C. Afterwards, cells were harvested by centrifugation at 8000 rpm for 10 min at 10 °C and washed twice with NaCl (8.5 g/l) solution. The pellet was re-suspended in NaCl (8.5 g/l) solution and then transferred to 300 µL tubes. The stocks were then frozen stored.

### Assessment of the cell growth phases

Three sets of 15 test tubes each containing 10 ml of sterile tryptone (Biokar) solution were used. Tubes of each set were labeled $t_0$, $t_2$, $t_4$, $t_6$, $t_8$, $t_{10}$, $t_{12}$, $t_{14}$, $t_{16}$, $t_{18}$, $t_{20}$, $t_{22}$, $t_{24}$, $t_{26}$ and $t_{28}$. Prior to the experiments, the stock frozen vial containing cells was thawed at room temperature. The culture (300 µl) was then transferred into 10 ml of nutrient broth (Oxford) and incubated at 37 °C for 24 hours. After, cells were then harvested by centrifugation at 8000 rpm for 10 min at 10 °C and washed twice with sterile NaCl (8.5 g/l) solution. The pellet was then re-suspended in 10 ml of sterilized solution containing NaCl (8.5 g/l) solution. After dilution, 100 µl was added to 100 ml of sterilized NaCl (8.5 g/l) solution, in each of the 15 tubes containing sterilized peptone solution. Cell suspensions in the 3 tubes coded $t_0$ were immediately analyzed. Those in tubes coded $t_2$, $t_4$, $t_6$ … $t_{28}$ were incubated for 2, 4, 6… 28 hours at 37 °C. The CFUs were counted after each incubation duration. The averages of the CFUs were calculated from the results of the triplicates and the Log(CFU) also calculated. The straight Log(number of CFUs) curve against storage duration was plotted and then assimilated as the cell growth curve. The cell growth phases were then assessed.

### Disinfectants and adsorbant substrates used

Two disinfectants were used: NaOCl, which belongs to the group of halogen derivatives and $H_2O_2$

belonging to the group of oxidants. The initial concentration of the $H_2O_2$ was 10 volumes. To count the surviving bacteria after disinfection treatment, sterile NaCl solution (8.5 g/l) was used as diluent. The disinfectant concentrations used ranged from 0.5 ‰ to 1.5 ‰ and from 5 ‰ to 15 ‰, for NaOCl and $H_2O_2$ respectively. The easier use of these two disinfectants in the drinking water treatment has justified their choice for this study.

The substrate used is high dense polyethylene. It differs from radical low dense polyethylene and linear low dense polyethylene by the molecular structure of its sparsely branched chains, and its relatively high resistance to shocks, high temperatures and ultraviolet [24, 25]. It is a plastic piping material obtained directly from the supplier and used in drinking water distributing.

**Adhesion protocol of *A. hydrophila* to polyethylene**

On the basis of previous studies, parallelepiped fragments of polyethylene with 13.28 cm² of total surface area suspended with inconsiderable diameter wire were immersed in triplicate in two sets A and B each in four flasks 250 ml Duran A1, A1', A1'' and B1, B1', B1'', A2, A2', A2'' and B2, B2', B2'', A3, A3', A3'' and B3, B3', B3'' and A4, A4', A4'' and B4, B4', B4'' each containing 99 ml of NaCl solution (8.5 g/l). Meanwhile, the controls were made and were coded $A_0$1, $A_0$2, $A_0$3, $A_0$4 and $B_0$1, $B_0$2, $B_0$3, $B_0$4 [26]. The whole was then autoclaved.

Prior to the experiments, stocks frozen vial containing *A. hydrophila* were thawed at room temperature. Then 100 µl of the culture was transferred into test tubes containing 10 ml of nutrient broth (Oxford) and incubated at 37 °C for 24 hours. Cells from a specific growth phase were then harvested by centrifugation at 8000 rpm for 10 min at 10 °C and washed twice with sterile NaCl solution (8.5 g/l). The pellets were then re-suspended in 50 ml of sterilized NaCl solution (8.5 g/l). After serial dilutions, the concentration of bacteria in each solution was adjusted to $6x10^8$ CFU/ml by reading the optical density at 600 nm using a spectrophotometer (DR 2800) followed by culture on agar [26]. 1 ml of the suspension was added to 99 ml of sterilized NaCl solution (8.5 g/l) contained in an Erlenmeyer flask.

The flasks, $A_0$1, A1, A1', A1'', $A_0$2, A2, A2', A2'', $A_0$3, A3, A3', A3'', and $A_0$4, A4, A4', A4'' were incubated under dynamic condition at a stirring speed 60 rev/min, using a stirrer (Rotatest brand). The flasks, $B_0$1, B1, B1', B1'', $B_0$2, B2, B2', B2'', $B_0$3, B3, B3', B3'' and $B_0$4, B4, B4', B4'' were incubated under static conditions. The incubation duration was 180 minutes for the Erlenmeyer flasks $A_0$1, A1, A1', A1''and $B_0$1, B1, B1', B1''. That of $A_0$2, A2, A2', A2''and $B_0$2, B2, B2', B2'' was 360 min. For the Erlenmeyer flasks coded $A_0$3, A3, A3', A3''and $B_0$3, B3, B3', B3'' and those coded $A_0$4, A4, A4', A4'' and $B_0$4, B4, B4', B4'', the incubation durations were 540 and 720 min respectively. All these incubations were done at laboratory temperature (25 ± 1 °C).

**Disinfection experiments**

After each incubation duration, fragments of polyethylene were drained for 10 seconds in a sterile environment created by the Bunsen burner flame and then introduced into test tubes containing 10 ml of diluted disinfectant of various concentrations. Fragments removed from flasks A1, A2, A3, A4 were introduced in disinfectant solutions of 0.5 ‰ NaOCl. Those removed from flasks B1, B2, B3 and B4 were introduced in 5 ‰ $H_2O_2$. Fragments removed from flasks A1', A2', A3', A4' and those removed from B1', B2', B3' and B4' were introduced into 1 ‰ NaOCl and 10 ‰ $H_2O_2$ solutions respectively. Similarly, those removed from flasks A1'', A2'', A3'', A4'', and from the flasks B1'', B2'', B3'' and B4'' were introduced into 1.5 ‰ NaOCl and 15 ‰ $H_2O_2$ solutions respectively.

Fragments of polyethylene flasks from $A_0$1, $A_0$2, $A_0$3, $A_0$4 and $B_0$1, $B_0$2, $B_0$3, $B_0$4 were introduced into 10 ml of sterile NaCl solution (8.5 g/l). After 30 min of incubation at room temperature and under static conditions, each fragment was then drained out under sterile conditions. Each fragment was then introduced into 10 ml of sterilized NaCl solution (8.5 g/l).The unhooking of adherent cells was performed by vortex agitation at increasing speeds for 30 seconds in three consecutive series of 10 ml sterilized NaCl solution (8.5 g/l).This technique allows the unhooking of maximum adhered cells [27, 28]. The total volume of the suspension containing the unhooked bacterial cells was 30 ml. The isolation and enumeration of unhooked cells was made by culture on ampicillin dextrin agar, by the method of surface spreading, followed by incubation on Petri dishes at 37 °C for 24 hours.

**Data analysis**

The variation of the abundances of adhered *A. hydrophila* in each experimental condition was illustrated by semi-logarithmic diagrams. Standard deviations were not fitted because the curves were too close. Spearman "r" Test correlation was used to assess the degree of correlation between the abundances of adhered *A. hydrophila* and other parameters considered. Kruskal-Wallis and Mann-Whitney tests were used to compare the mean abundance of cells adhered from one experimental condition to another.

Adhesion speeds of *A. hydrophila* on polyethylene were assessed constructing linear regression of adhered *A. hydrophila* after each incubation duration of three hours by means of Excel program. The ratio slope of the linear regression and three hours correspond to adhesion speeds of *A. hydrophila* per hours. These results are expressed as adhered cells/cm²/hours.

The data from absorption experiments were analyzed using the Freundlich model. This isotherm was chosen because of the number and the relevance of the information it provides on the real adsorption mechanisms on one hand and its remarkable ability to match doses of adsorption on the other hand [29, 30]. The Freundlich isotherm is described by the following equation [29, 30]:

$$C_s = K_f . C^{1/n}$$

With $C_s$; the quantity of cells adsorbed in the presence of disinfectant, $C$; the concentration of cells adsorbed in the absence of disinfectant, $K_f$; the Freundlich coefficient adsorption which is connected to the adsorption capacity, $1/n$; coefficient linearity, and $n$ being the intensity of adsorption. Here, *Cs* is expressed as number of adherent cells/disinfectant concentration and *C*, the number of adherent cells/cm² of polyethylene. Constructing linear regression log *Cs* versus log *C*, resulting in a line of slope l/n which intercepts the y-axis log $K_f$.

## Results and Discussion

**Cell growth curve**

The growth of *A. hydrophila* in non-renewed peptone liquid medium explained a hyperbolic curve of 4 phases: a lag growth phase from 0 to 2 hours, an exponential growth phase from 2 to 13 hours, a stationary growth phase from 13 to 22 hours, and a decline growth phase begins from the 22th hour (Figure 1).

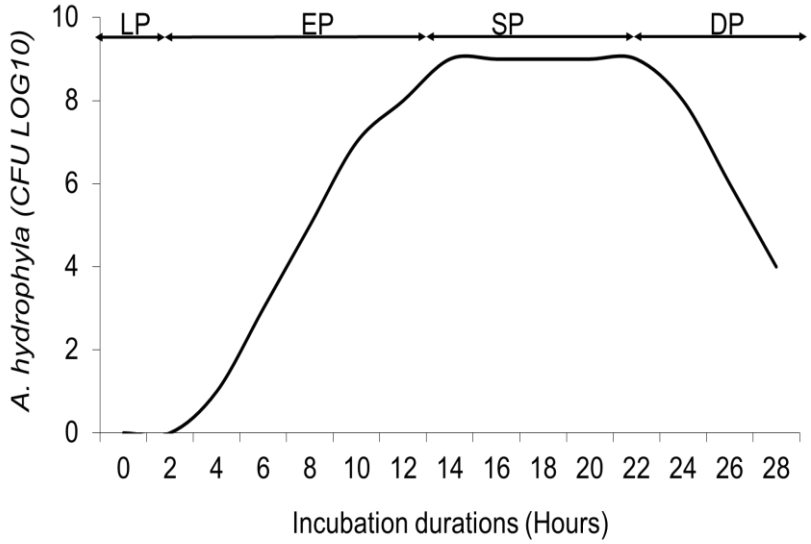


**Figure 1: Growth curve of *A. hydrophila* (LP: Lag growth phase, EP: Exponential growth phase, SP: Stationary growth phase, DP: Decline growth phase)**

**Adhesion kinetics in static and dynamic conditions**

The hourly cell adhesion speeds on the considered substrate was assessed in static and dynamic conditions. It varied from one growth phase to another. Under static condition, adhesion speeds varied from 0.002 to 0.870 cell/h. Under dynamic condition, it varied from 0.005 to 0.930 cell/h. In both conditions, the lowest cell adhesion speeds were registered with cells harvested from the stationary growth phase whereas the highest were noted with cells harvested from the lag growth phase (Table 1).

**Table1: Hourly adhesion speeds (and regression coefficient) of *A. hydrophila* with respect to growth phases under static and dynamic conditions.**

| Cellular growth phases | Adhesion speeds ($h^{-1}$) (regression coefficient) | |
|---|---|---|
| | Static | Dynamic |
| Lag | 0.8704 (0.2669) | 0.9302 (0.2601) |
| Exponential | 0.0736 (0.3096) | 0.1805 (0.3277) |
| Stationary | 0.0019 (0.2700) | 0.0050 (0.3184) |
| Decline | 0.1801 (0.2008) | 0.7890 (0.2022) |

**Abundance of *A. hydrophila* adhered to polyethylene after NaOCl disinfection**

The densities of adhered *A. hydrophila* ranged from 0 to 256 CFU/cm² after the action of NaOCl. The maximum abundance of adhered *A. hydrophila* was recorded under dynamic condition, in the presence of 0.5 ‰ NaOCl and this after 720 min with cells harvested from the lag growth phase. Adhered *A. hydrophila* was sometimes been totally decimated by NaOCl. This result was observed during the decline phase in static and dynamic conditions in the presence of three concentrations of NaOCl, at the end of all incubation durations. The same observation was made for adhered *A. hydrophila* during the stationary growth phase, in the presence of 1.5 ‰ NaOCl at 180 and 360 min incubation durations (Figure 2).

With cells coming from the lag phase, the abundance of *A. hydrophila* adhered to the control substrate varied throughout from 105 to 1799 CFU/cm² and had always been superior to those of fragments tested for disinfection. In addition, they increase with the incubation duration. Maximum cell density was recorded after an adhesion test of 720 min under dynamic condition when studying the impact of NaOCl. After action of NaOCl, the densities of adhered *A. hydrophila* ranged from 1 to 256 CFU/cm². The effectiveness of NaOCl decreased with the length of the adhesion duration test. Maximum cell abundance was recorded in the presence of 0.5 ‰ NaOCl after an adhesion test of 720 min in dynamic regime. The lower density of adhered cells was observed in the presence of 1.5 ‰ NaOCl with cells coming from the adhesion tests of 180 min under static condition (Figure 2).

Abundances of *A. hydrophila* adhered to control substrate during the exponential growth phase were also higher than those fragments tested for disinfection in the lag growth phase. They generally fluctuated between 12 and 313 CFU/cm². After disinfection test, it was noted that the effectiveness of NaOCl decreased when the duration of adhesion test increased. Abundance of adhered *A. hydrophila* ranged between 3 and 59 CFU/cm² (Figure 2).

With cells coming from the stationary growth phase, abundance of *A. hydrophila* adhered to the control substrate varied from 2 to 13 CFU/cm². They remained higher than those of fragments tested for disinfection. After disinfection test, abundances of adhered *A. hydrophila* ranged between 0 and 3 CFU/cm². As the duration of adhesion test increased, it was noted that the effectiveness of NaOCl decreased. The maximum density of *A. hydrophila* adhered to the polyethylene was recorded in the presence of 0.5 ‰ NaOCl after 720 min incubation duration and under dynamic and static conditions. The lower density of adhered cells was observed in the presence of 1.5 ‰ NaOCl after 180 and 360

min incubation durations under static and dynamic conditions (Figure 2). Abundances of *A. hydrophila* adhered to the control substrate during the decline growth phase varied from 0 to 4 CFU/cm². Adhered cells after the action of NaOCl were almost few (Figure 2).

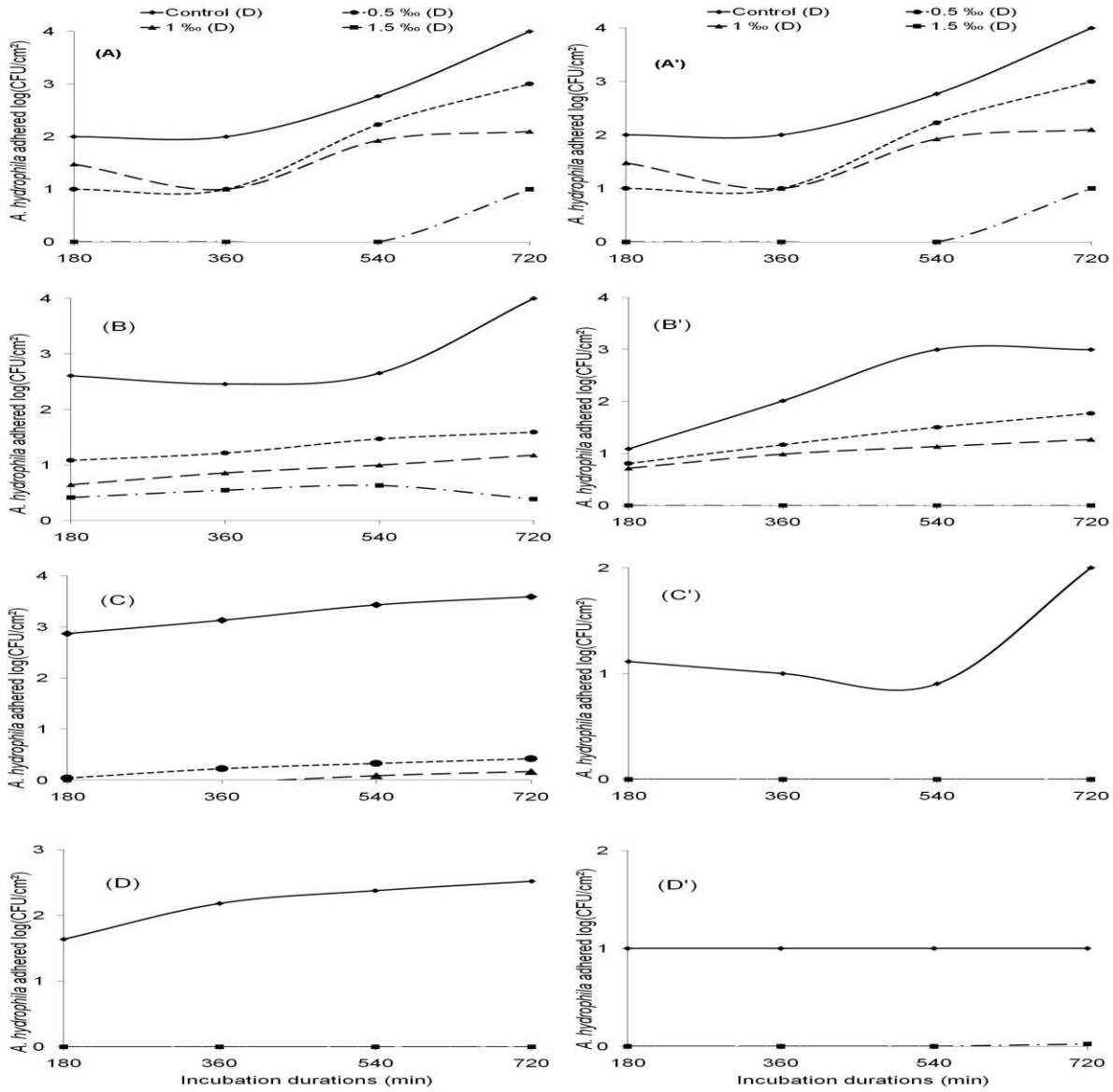


**Figure 2: Temporal evolution of adhered *A. hydrophila* under static and dynamic conditions, and after the action of NaOCl (A, A': Lag phase; B, B': Exponential phase; C, C': Stationary phase, D, D': Decline phase).**

### Abundance of *A. hydrophila* adhered to polyethylene after $H_2O_2$ disinfection

The densities of adhered *A. hydrophila* ranged from 5 to 2469 CFU/cm² after the action of $H_2O_2$. The maximum abundance of adhered *A. hydrophila* was recorded during the stationary cell growth phase under static condition, in the presence of 5 ‰ $H_2O_2$ and this after 720 min incubation duration (Figure 3). Adhered *A. hydrophila* has been sometimes always partially decimated by $H_2O_2$.

With cells coming from the lag growth phase, the abundance of *A. hydrophila* adhered to the control substrate increase with the increasing of the incubation duration. The highest cell density was recorded after 720 min of adhesion test under dynamic condition. After action of $H_2O_2$, the densities of adhered *A. hydrophila* ranged from 5 to 120 CFU/cm². The effectiveness of $H_2O_2$ decreased with the duration of the adhesion test. The highest cell abundance was recorded in the presence of 5 ‰ $H_2O_2$

after 720 min of adhesion test under dynamic regime. The lower density of adhered cells was observed in the presence of 15 ‰ $H_2O_2$ after 180 min of an adhesion test under static and dynamic conditions (Figure 3).

Abundances of *A. hydrophila* adhered to control substrate during the exponential growth phase fluctuated between 288 and 909 CFU/cm². The highest cell abundance was recorded under dynamic condition. After disinfection test, it was noted that the effectiveness of $H_2O_2$ decreased when the duration of adhesion test increased. Abundance of adhered *A. hydrophila* ranged between 127 and 661 CFU/cm² (Figure 3).

With cells harvested from the stationary growth phase, abundance of *A. hydrophila* adhered to the control substrate remained higher than those fragments tested for disinfection. The highest cell density was recorded after an adhesion test under static condition. After disinfection test, abundances of adhered *A. hydrophila* ranged between 50 and 2469 CFU/cm². As the duration of adhesion test increased, it was noted that the effectiveness of $H_2O_2$ decreased. The highest density of *A. hydrophila* adhered to the polyethylene was recorded in the presence of 5 ‰ $H_2O_2$ after 720 min of adhesion test under static condition. The lowest was observed after 180 min of adhesion test under static condition in the presence of 15 ‰ $H_2O_2$ (Figure 3).

Abundances of *A. hydrophila* adhered to the control substrate during the decline cell growth phase varied from 43 to 1669 CFU/cm². The maximum abundance was recorded under dynamic condition after 720 min of adhesion test. Abundances of adherent cells ranged between 20 and 1474 CFU/cm² after disinfection test. The maximum abundance of *A. hydrophila* adhered to the polyethylene was recorded in the presence of 5 ‰ $H_2O_2$ after 720 min whereas the lowest was registered in the presence of 15 ‰ $H_2O_2$ after 180 min. Both abundances were noted under dynamic condition (Figure 3).

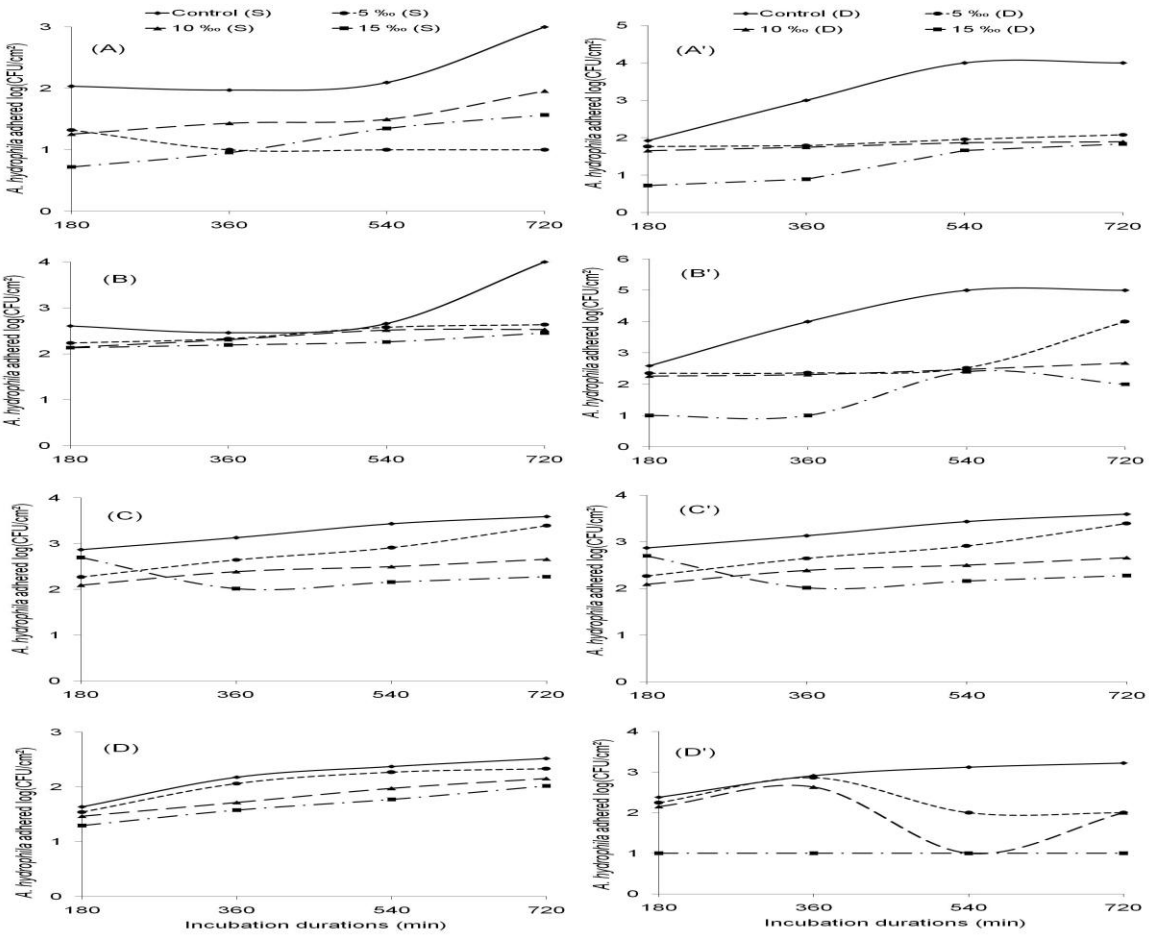


**Figure 3: Temporal evolution of adhered *A. hydrophila* under static and dynamic conditions, and after the action of $H_2O_2$ (A, A': Lag phase; B, B': Exponential phase; C, C': Stationary phase, D, D': Decline phase).**

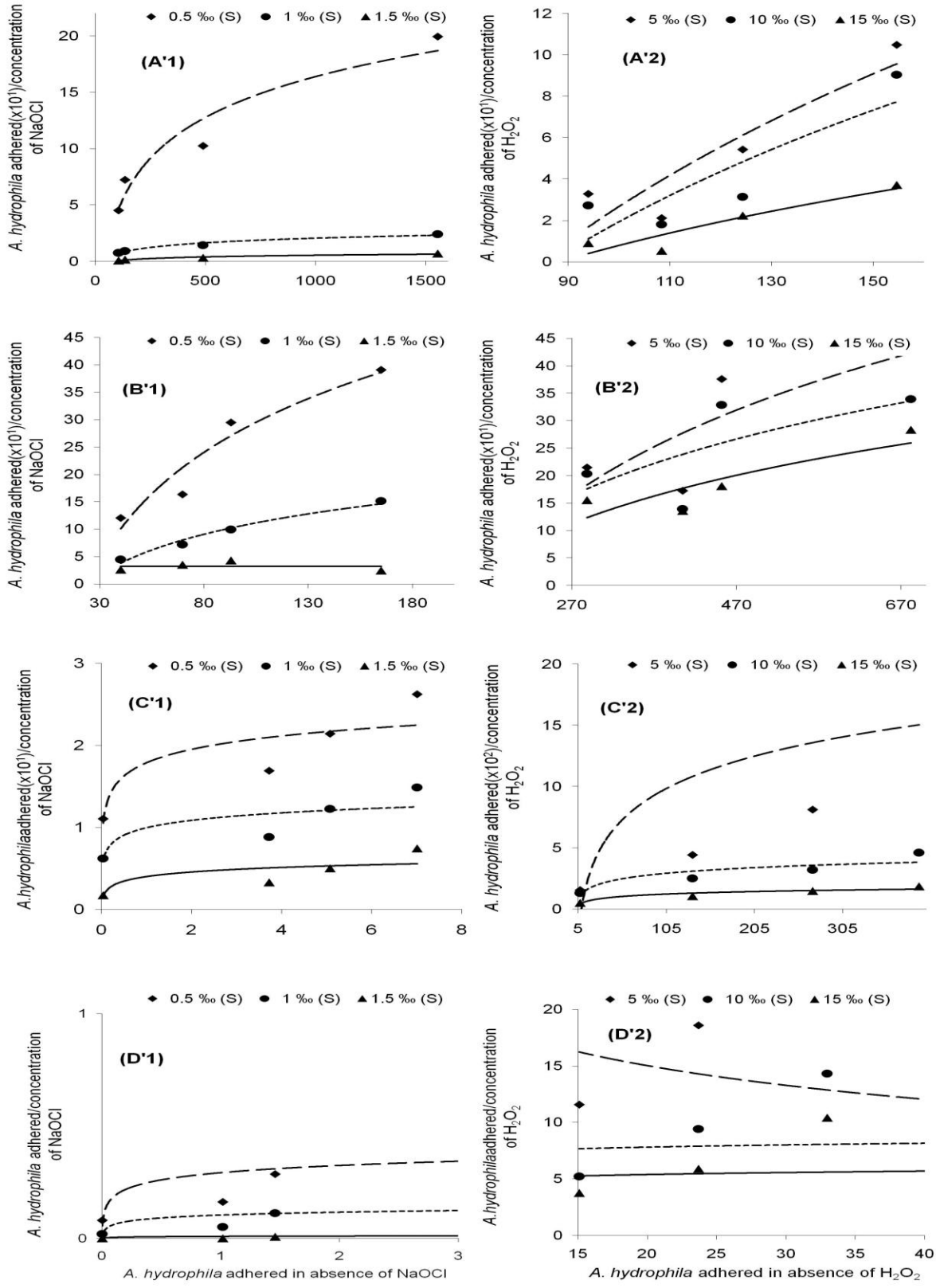


**Figure 4: Freundlich Isotherms for *A. hydrophila* under static condition in the presence of NaOCl and $H_2O_2$ [lag (A'1, A'2), exponential (B'1, B'2), stationary (C'1, C'2), and decline (D'1, D'2)]**

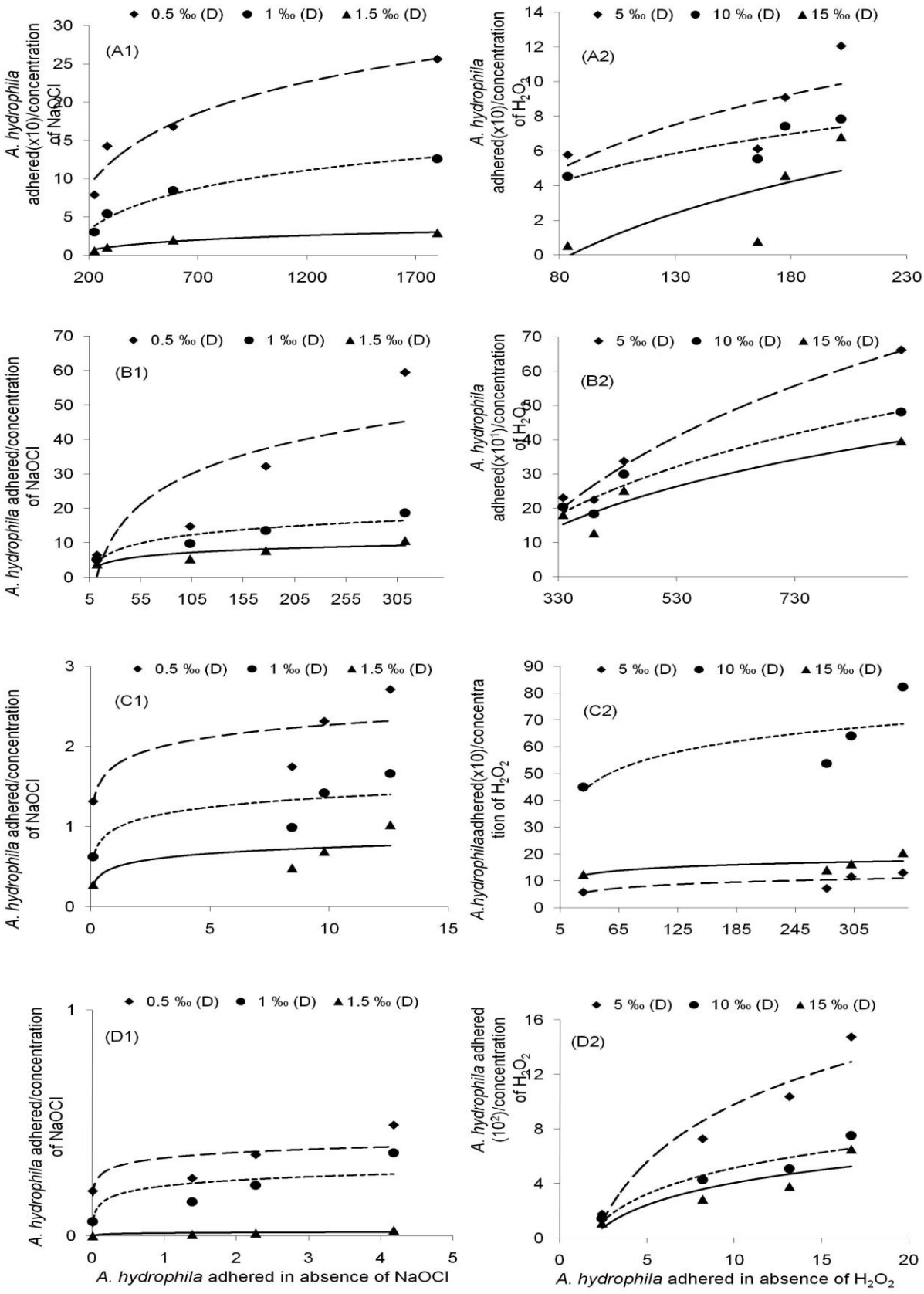


**Figure 5: Freundlich Isotherms for *A. hydrophila* under dynamic condition in the presence of NaOCl and $H_2O_2$ [lag (A1, A2), exponential (B1, B2), stationary (C1, C2), and decline (D1, D2)]**

**Freundlich isotherms of *A. hydrophila***

Freundlich isotherms were constructed by considering the number of *A. hydrophila* adhered to the substrate and subjected to the test of disinfection, and that obtained without exposure to disinfectants for each stage of cell growth and each experimental condition. They are shown in figures 4 and 5. It can be noted that whether *A. hydrophila* is in a static or dynamic incubation condition or coming from a definite cell growth stage, the appearance of the isotherms differ from one disinfectant to another. The linearity coefficient *l/n* which is related to the adsorption intensity ranged from 0.001 to 31.423 and from 0.002 to 16.286 for NaOCl, from 0.786 to 1544.335 and from 0.024 to 533.932 for $H_2O_2$ respectively under dynamic and static incubation conditions. For the whole disinfectant concentrations of NaOCl used, the adsorption coefficient $K_f$ which is related to the adsorption capacity ranged between 1 and 403, and between 1 and 1627 adhered *A. hydrophila* respectively under static and dynamic incubation conditions; For the whole disinfectant concentrations of $H_2O_2$ used, it ranged between 0 and 148, and between 0 and 813 adhered *A. hydrophila* respectively (Table 2).

The lowest adsorption coefficient after NaOCl treatment was obtained with cell harvested from the decline and stationary cell growth phases. The same observations were made with $H_2O_2$ (Table 2). When considering each experimental condition or cells harvested from each growth stage, the adsorption coefficient of *A. hydrophila* adhered to polyethylene was relatively higher after NaOCl treatment than that after $H_2O_2$ (Table 2). It was also noted that for the whole cell growth phases and the whole disinfectant concentrations used, the adsorption coefficient values were relatively higher under static than dynamic incubation condition (Table 2).

**Correlation between the abundances of adhered *A. hydrophila* and incubation durations or disinfectant concentrations**

Spearman "r" correlation coefficients between the abundances of adhered *A. hydrophila* and incubation durations for each concentration disinfectant and under each experimental condition were assessed and are presented in (Table 3). It is noted that the increase of the incubation durations caused a significant ($P<0.01$) decrease of the efficiency of each of the disinfectant concentration. This could result in higher abundances of adhered *A. hydrophila* as the duration of the cell adhesion process increased.

Spearman "r" correlation coefficients between abundances of adhered *A. hydrophila* and disinfectant concentrations for each incubation duration and under each experimental condition were also assessed. Under static as well as dynamic condition, it was noted that the effectiveness of NaOCl and $H_2O_2$ on *A. hydrophila* adhered to polyethylene increased, which leads to a significant decrease ($P<0.01$) in the abundance of bacteria adhered after disinfection treatment.

The degrees of relationship between the disinfectant concentrations and abundances adhered *A. hydrophila* harvested from each growth stage were also assessed. It resulted that an increase of the disinfectant concentration significantly increased ($P<0.01$) the abundance of *A. hydrophila* adhered to the substrate, with cell harvested from each growth phase.

**Comparison amongst abundances of adhered *A. hydrophila* harvested from different cell growth stages**

The H test of Kruskal-Wallis was performed in order to compare the abundances of adhered *A. hydrophila* harvested from different cell growth stages and considering each disinfectant concentration. It showed that there was an overall significant difference ($P<0.05$) between the mean abundances of *A. hydrophila* adhered to polyethylene for each disinfectant concentrations at different growth stages. The pair two by two comparisons of the mean abundances were then performed using the U test of Mann-Whitney. It was noted that at each cell growth stage, there was a significant difference ($P<0.05$) between the mean abundances of adhered *A. hydrophila* after action of various concentrations of NaOCl with cells coming from each the growth phase. With different concentrations of $H_2O_2$, a significant difference ($P<0.05$) was observed only with cells harvested from lag growth phase (Table 4).

The growth curve of *A. hydrophila* obtained shows a lag growth phase from 0 to 2 hours, an exponential growth phase from 2 to 13 hours, a stationary growth phase from 13 to 22 hours, and a

decline growth phase begins from the 22$^{th}$ hour. It described a hyperbola of four phases. Bacterial growth is an orderly increment of all the components of the bacterium [31]. It leads to an increase in the number of bacteria. During growth, there is, on one hand, a depletion of nutrients in the culture medium and, on the other hand, an enrichment of products of metabolism, that are toxic. During the lag growth phase, the growth rate is nil. Bacteria adapt and synthesize the enzymes necessary to metabolize new substrates. The exponential growth phase corresponds to the period of nutrient utilization and duplication of cell number. The stationary growth phase is the period when the growth rate becomes nil. In fact, the bacteria multiply compensating those who die. The decline growth phase is the time when all food resources are exhausted. There is accumulation of toxic metabolites. There is a decrease of viable organisms and an occurring of cell lysis by the action of endogenous proteolytic enzymes. However, there is a persistant growth leading to the release of substances during lysis (cryptic growth) [31].

The adhesion of microorganisms to surfaces is the first step in biofilm formation which is a form of microbial life in aquatic environments [32]. The latter is a source of biocontamination problems in various domains such as health, environment, food industry, water purification [30, 33, 34]. Adhesion is governed by physico-chemical interactions of van der Waals type and Lewis's acid-base. Fluctuating of speeds of adhesion of *A. hydrophila* observed during different stages of growth in static and dynamic conditions could be explained by changes in the physiology of bacteria in each growth stage [35, 36].

This study showed that whatever the experimental condition, the abundances of adhered *A. hydrophila* at all stages of growth are generally lower in the presence of NaOCl and $H_2O_2$ than in the absence of these disinfectants. Unlike antibiotics, the mode of action of disinfectants is characterized by a lack of specificity, and remains partially understood. Three possible action phases are; the binding to the bacterial wall, conditioned by a disinfectant concentration and the Brownian movement of bacteria [37]. The phenomenon is chemical or electrical. The penetration through the wall and the membrane is conditioned by solubility and ionization of steric hindrance. The action itself, can affect different targets including the cytoplasmic membrane and organelles [25]. In addition, the standard AFNOR T 72.101 defines disinfection as an operation that permits the elimination or killing of micro-organisms and/or inactivates undesirable viruses carried by contaminated inert environments, based

on the already fixed objectives on a momentary resultant [38]. The result of this operation is limited to microorganisms present at the time of the operation. Considering separately, each experimental condition, it was noted on one hand that the increase in incubation durations lead to a very significant decrease ($P<0.01$) in the effectiveness of each disinfectant concentrations. This leads to a rise in the abundances of adhered *A. hydrophila*. In fact, a biofilm can develop within hours, allowing bacteria therein to become resistant to external agents causing any eventual contamination [39, 40]. On the other hand, for each incubation duration, the action of NaOCl and $H_2O_2$ on *A. hydrophila* adhered to polyethylene increased significantly ($P<0.01$). The action of these disinfectants is explained by the fact that they are chemically more reactive molecules on biofilms [41]. Furthermore, this variation of the reaction of *A. hydrophila* with disinfectants may be related to changes in their surface due to a change in their growth stage [20]. Whether in the presence of NaOCl or $H_2O_2$, a significant difference ($P<0.05$) between the mean densities of adhered *A. hydrophila* to different stages of growth was recorded. This difference could be due to a nutritional insufficiency that suffered *A. hydrophila* and that could cause a change in the growth rate [42, 43, 44]. Same at each growth stage there was a significant difference ($P<0.05$) between the mean abundances of *A. hydrophila* adhered after action of various concentrations of NaOCl. It is important to remember that the bacteria in a biofilm have characteristics very different from their planktonic counterparts including the production of exopolymers [45], a significant increase in their resistance to antimicrobial agents and environmental stress [46, 47]. The exopolymeric matrix that presents itself as a mechanical barrier, by reducing the rate of penetration of compounds from the environment through the biofilm protects the bacterial cells enclosed in the biofilm. This explains the fact that the increase in the concentration of disinfectants for each growth stage resulted in a significant increase ($P<0.01$) in the abundance of adhered *A. hydrophila* to the substrate.

No significant difference between the mean densities of adhered *A. hydrophila* under static condition and those obtained under dynamic condition has been noted ($P>0.05$). In fact, the conservation of water quality is even easier than the residence time in the distribution network is short. The average residence time in the distribution network can be in the order of a few days, but some amounts of water can stagnate over ten days in areas of the distribution network where the flow is low or when

water demand is almost nil (during holiday periods for example). A sudden increase in the flow rate can cause tearing of the biofilm and therefore a transient but significant deterioration of water quality in distribution networks [48]. It has been indicated that the stagnation or slow flow speeds favors corrosion and biofilms deposits or conditioning film by adsorption of macromolecules which can lead to the modification of the surface properties of the substrate, and therefore, promote bioadhesion of the pipe surfaces. These deposits appear as soon as the water speed is less than 0.01 $ms^{-1}$ and disappear beyond 0.1 $ms^{-1}$[49]. However, for a given biofilm, a turbulent flow is also beneficial in promoting the transport of nutrients and micro-organisms [50]. This allows the renewal of the environment and improves the stability of biofilms [51].

Whether each experimental condition or each growth stage is considered, the adsorption coefficient $K_f$ of *A. hydrophila* adhered to polyethylene is relatively higher in the presence of NaOCl than in the presence of $H_2O_2$. This could be explained by the fact that NaOCl is made up of more chemically reactive molecules on biofilms than $H_2O_2$ [41]. In addition, considering *A. hydrophila* at a growth stage in the presence of a given disinfectant, the adsorption coefficient $K_f$ is relatively higher in static than dynamic condition. *A. hydrophila* adhered to polyethylene in dynamic condition is more sensitive to both disinfectants than that adhered under static condition. This could be explained by the structure of the adhered bacteria which depend on hydrodynamic regime [52].

## Conclusion

Abundances of *A. hydrophila* adhered at all cell growth stages is relatively low in the presence of NaOCl and $H_2O_2$ than in the absence of these disinfectants, whatever the experimental condition. In the presence of NaOCl as well as in the presence of $H_2O_2$, there is a significant difference between the mean densities of *A. hydrophila* adhered to different growth stage. The effectiveness of each concentration of NaOCl and $H_2O_2$ on *A. hydrophila* adhered to polyethylene decreased as the duration of the adhesion test increased. Although the adsorption coefficient obtained from Freundlich isotherms was relatively higher in static than in dynamic condition, no significant difference was observed between the mean abundances of adhered *A. hydrophila* under these two experimental conditions. Overall, NaOCl seems more effective on *A. hydrophila* adhered to the polyethylene than $H_2O_2$. Furthermore, *A. hydrophila* adhered to the polyethylene in dynamic condition seems more sensitive to the two disinfectants than that adhered in static condition.

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**Table 2: Values of adsorption coefficient $K_f$ (adhered *A. hydrophila* /ml of disinfectant) and linearity coefficient *l/n* of isotherms under static and dynamic conditions.**

| Disinfectant and its concentrations | | Experimental condition, cell growth phase and Freundlich coefficient | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Static | | | | | | | | Dynamic | | | | | | |
| | | Adsorption ($K_f$) | | | | Linearity (*ln*) | | | | Adsorption ($K_f$) | | | | Linearity (*ln*) | | | |
| | | Lag | Expo. | Stat. | Decl. | Lag | Expo. | Stat. | Decl. | Lag | Expo. | Stat. | Decl. | Lag | Expo. | Stat. | Decl. |
| NaOCl | 0.5‰ | 2 | 403 | 3 | 1 | 31.42 | 0.27 | 0.40 | 0.06 | 3 | 1627 | 3 | 1 | 16.29 | 1.26 | 0.34 | 0.02 |
| | 1‰ | 1 | 148 | 3 | 1 | 11.29 | 1.68 | 0.18 | 0.02 | 298 | 55 | 3 | 1 | 2.51 | 0.45 | 0.19 | 0.02 |
| | 1.5‰ | 403 | 55 | 1 | 1 | 2.29 | 1.12 | 0.07 | 0.00 | 3 | 20 | 1 | 1 | 0.26 | 1.18 | 0.04 | 0.00 |
| $H_2O_2$ | 5‰ | 2 | 8 | 3 | 0 | 1.43 | 4.21 | 3.14 | 1544.33 | 2 | 2 | 1 | 0 | 35.44 | 6.8 | 4.86 | 533.93 |
| | 10‰ | 3 | 1 | 5 | 0 | 12.09 | 3.80 | 6.31 | 1306.37 | 5 | 1 | 4 | 0 | 31.40 | 22.12 | 1.05 | 397.67 |
| | 15‰ | 148 | 4 | 6 | 0 | 27.66 | 2.93 | 0.79 | 369.44 | 813 | 2 | 4 | 0 | 14.78 | 7.69 | 0.02 | 168.57 |

**Table 3: Spearman "r" correlation coefficients between the abundances of adhered *A. hydrophila* and incubation durations for each concentration of disinfectant and each experimental condition.**

| Experimental condition | Disinfectant concentrations | | | | | |
|---|---|---|---|---|---|---|
| | NaOCl | | | $H_2O_2$ | | |
| | 0.5 ‰ | 1 ‰ | 1.5 ‰ | 5 ‰ | 10 ‰ | 15 ‰ |
| **Static** | 0.273 | -0.405** | -0.533** | 0.003 | -0.079 | -0.021 |
| **Dynamic** | -0.542** | -0.700** | -0.710** | 0.066 | 0.391* | -0.568** |

** P <0.01 ddl=15

**Table 4: Comparison amongst abundances of *A. hydrophila* harvested from different cell growth stages in the presence of each disinfectant**

| Cell growth phases | *A. hydrophila* | |
|---|---|---|
| | **NaOCl** | **$H_2O_2$** |
| **Lag** | P=0.007* | P=0.034* |
| **Exponential** | P=0.000* | P=0.114 |
| **Stationnary** | P=0.000* | P=0.185 |
| **Decline** | P=0.015* | P=0.963 |

* P<0.05 ddl=92