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## Effects of Copper Sulfate and Zinc Sulfate on Cell Adhesion of *Staphylococcus aureus* and *Aeromonas hydrophila* Stemming from Different Cell Growth Phases in Aquatic Microcosm

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### Abstract

This study aimed at evaluating the effect of different concentrations of zinc sulfate and copper sulfate on bacterial adhesion *A. hydrophila* and *S. aureus* polyethylene at different stages of growth, different exposure times and monitor the pH and conductivity during the adhesion test. The analyzes showed that the maximum abundance of adhered cells were obtained in the lag growth phase and exponential growth phase. A negative and significant difference was observed between the abundance of adherent cells and concentrations of heavy metal salts. Indeed, in every condition of experience, it was noted that increasing the incubation period lead to a significant increase in abundance of bacterial cells adhered to polyethylene, so the increase in the concentrations of heavy metal salts significantly decreases the abundance of adherent cells. These results suggest that the incubation period, growth phase and the concentrations of heavy metal salts have an influence on the adhesion of *S. aureus* and *A. hydrophila* in polyethylene. The use of heavy metals with small concentration can be an additional process, in reduction of planktonic cells using adhesion process.

**Keywords:** Cell Growth Phases; Bacteria Adhesion; Polyethylene; Heavy Metals

## Introduction

Bacteria are indicators of fecal contamination of water. They are generally in a planktonic state and can adhere to suspended matter or substrates in the environment. They can then multiply on the surfaces of these substrates, forming biofilms subsequently. These highly hydrated microcolonies can have harmful consequences. Indeed, inside the pipes carrying water, the microbial densities on the walls of the materials used in the distribution of water intended for human consumption can reach  $10^8$  CFU/cm<sup>2</sup>. In addition, the adhesion of microorganisms to surfaces, a significant factor in microbial resistance to disinfectants depends on the bacterial species considered, and also on the origin of the strain and the conditions of its growth [1]. Bacteria adhered and/or in biofilms constitute a recurring source of contamination and their elimination is relatively difficult [2]. In addition, these biofilms are escape routes for microorganisms in the face of disinfectants. Some authors have revealed that bacterial and yeast biofilms have been frequently observed on the internal walls of drinking water pipes. In the aquatic environment, bacteria have the ability to form complex ecosystems called biofilms either on the surface of the water or adhered to inorganic supports submerged in water. These biofilms are particularly observed in drinking water distribution networks where bacteria adhere to distribution pipes. This biomass is responsible for the contamination of circulating water by the resuspension of adherent bacteria. The adhesion of bacteria is a fundamental step in the process of biofilm formation. It depends on the properties inherent in the bacteria itself [3], such as the presence of pili or flagellum and also depends on the nature of the substrate or the dissolved salts [4]. The use of these factors thus offers a means of combating microbiological pollution of water intended for consumption. Microbiological pollution, due to the presence in the water of potentially pathogenic microorganisms (protozoa, viruses, bacteria), are responsible for the deterioration of water quality. The presence in the water intended for consumption of these germs is unacceptable characteristic because water of poor physico-chemical and biological quality responsible for water-borne diseases [5]. Reducing and inhibiting the load of bacteriopolutants in the water is therefore a health and vital necessity. Industrial wastewater typically dumped into waterways contains levels of heavy metals that can pollute the environment once it is discharged into nature. These metals include As, Cr, Cu, Zn, Al, Cd, Pb, Fe, Ni, Hg, and Ag [6]. Various products are used to destroy microorganisms present in water. Certain

metals are emitted into the environment in hazardous amounts. Heavy metals are not only harmful to humans, they can often have toxic effects on bacteria, much like antibiotics [7]. Various studies have shown that heavy metals can have adverse effects on bacterial growth and activity in aquatic environments [8,9]. The effect of these metals on bacteria varies according to the concentrations used, thus they can manifest themselves by an inhibition of bacterial growth in the case of zinc or even up to a bactericidal effect in the case of copper.

Despite this extensive work carried out on the bactericidal and bacteriostatic activity of heavy metals, few data are available on the actual effects of heavy metals on the adhesion to polyethylene of opportunistic bacteria. In addition, the influence of the growth phase corresponding to a precise physiological state in bacterial species on the adhesion mechanisms of these is very little documented. Likewise, little information is available as to the phenomenon of competition between different bacterial species at the same growth phases, subjected to stress caused by heavy metals for the acquisition of a site for attachment to a support. On the other hand, with the exception of some studies on the *Staphylococcus aureus* and *Aeromonas hydrophila* species which have shown that, the presence in drinking water of these bacteria is one of the causes of gastrointestinal diseases [10]. It characterized by watery diarrhea, very few studies have put the emphasis on their adhesion as a means of resistance to stress caused by chemicals in the water. The present study therefore aimed at evaluating in microcosm, the effects of copper sulphate and zinc sulphate on the adhesion of the cells of the bacteria *S. aureus* and *A. hydrophila* at different stages of growth on fragments of polyethylene immersed in the water. Studies have been conducted to elucidate the impact of a zinc sulfate and copper sulfate concentration range on the adhesion of *S. aureus* and *A. hydrophila* on polyethylene, to evaluate the influence of the different growth phases of bacterial growth on bacterial adhesion and to determine the influence of some abiotic parameters of water on this studied microbiological process.

## Materials and Methods

### Choice of substrate used and bacteria species for experimentation

The choice of high-density polythene as adsorbent substrates was based to its relatively high resistance to shocks, high tempera-

tures and ultra-violet rays [11]. Its wide use in household utensils (barrels, buckets, cans) for water supply and the drinking water distribution networks. In addition, the hydrophobic character of polythene makes it a favorable adhesion of bacteria material [2]. The bacteria used for the adhesion test were *S. aureus* and *A. hydrophila*. These bacteria isolated in well water are known for their importance in public health [12]. *Staphylococcus aureus* is a gram-positive commensal bacterium causing nosocomial infections [13]. Isolation of *Staphylococcus aureus* was performed using Mannitol agar medium, incubated at 37 °C for 24 to 48 hours. The bacterium *A. hydrophila* was isolated from well water using the membrane filtration technique, on ampicillin-dextrin agar culture medium [14]. The cells of *A. hydrophila* are anaerobic facultative, non-sporulated, Gram-negative bacilli.

### Preparation of heavy metal salt solutions

Solutions of  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  were prepared from copper sulfate pentahydrate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) and zinc sulfate monohydrate ( $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$ ). The masses 7.54 mg, 10.04 mg, 12.56 mg of copper sulphate and 9.58 mg, 14.8 mg, 19.74 mg of zinc sulphate were introduced respectively into 2L of distilled water each. The dissolution of these salts in distilled water leads to the formation of  $\text{SO}_4^{2-}$ ,  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  ions according these equations:



All the solutions thus prepared were sterilized in the autoclave at 121°C. The ions values of heavy metals ( $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ ) were set on the basis of drinking water standards [5,12]. For each of the two heavy metal salts used during this study, three ranges of concentration values were chosen (Table 1).

	Heavy metals used					
	$\text{CuSO}_4$			$\text{ZnSO}_4$		
Concentrations (mg / L)	1.5	2	2.5	2	3	4
$\text{Cu}_2^+$	$6.0 \cdot 10^{-3}$	$8.0 \cdot 10^{-3}$	$1.0 \cdot 10^{-2}$	-	-	-
Ions $\text{SO}_4^{2-}$	$6.0 \cdot 10^{-3}$	$8.0 \cdot 10^{-3}$	$1.0 \cdot 10^{-2}$	$1.1 \cdot 10^{-2}$	$1.7 \cdot 10^{-2}$	$2.2 \cdot 10^{-2}$
$\text{Zn}_2^+$	-	-	-	$1.1 \cdot 10^{-2}$	$1.7 \cdot 10^{-2}$	$2.2 \cdot 10^{-2}$

**Table 1:** Values of the concentrations of dissolved ionic species.

### Assessment of the cell growth phases

For each bacterial species, 3 sets of 15 tests tubes each containing 10 mL of sterile peptone water were used. Tubes in each series

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  $t_{28}$  [11]. Before the experiments, 300  $\mu\text{L}$  of a cell suspension kept cold in glycerol diluted to the third, were thawed at room temperature for 10 minutes then transferred to 10 mL of nutrient broth (Oxford) and incubated at 37 °C for 24 hours. Subsequently, the cells were harvested by centrifugation at 8000 rpm for 10 minutes at 100°C, and washed twice with sterile NaCl solution (8.5g/L). The supernatant was resuspended in 10 mL of sterile NaCl solution (8.5g/L). After dilution, a volume of 100  $\mu\text{L}$  of the suspension obtained was added to 100 mL of the sterile NaCl solution. Then 100  $\mu\text{L}$  of the new suspension were introduced into each of the 15 tubes containing the sterilized solution of peptone. The bacterial suspensions contained in the 3 tubes coded  $t_0$  were immediately analyzed. Those contained in the tubes coded  $t_2, t_4, t_6 \dots t_{28}$  were incubated at 37 °C for 2, 4, 6... 28 hours respectively [11]. Bacteriological analyses were then performed, and Colony Forming Units (CFU) were counted after each incubation period. The experiment being carried out in triplicate, the mean of the CFU was calculated after each incubation period as well as the Log (CFU). The Log curve (CFU) depending on the incubation period was plotted. The durations of the cell growth phases of the two bacterial strains were then determined.

### Experimental protocol

The pure colonies were taken on agar sloped in the test tubes using a sterile platinum loop and introduced into sterile physiological water (0.85% NaCl). The homogeneous bacterial suspension obtained after vortexing was then centrifuged at 8000 rev/ for 10 minutes at 10°C. Subsequently, 50  $\mu\text{L}$  of the suspension obtained was introduced into 10 ml of peptone water and incubated at 37 °C. In order to maintain the bacteria in a well-defined growth phase. After incubation, 1 mL of this suspension was taken and introduced into 10 mL of physiological water and then stirred in a vortex. The bacterial suspension obtained constituted the stock solution. Once the bacterial suspension was homogenized, the concentration in the stock solution was adjusted to  $2 \times 10^8$  CFU/mL by reading the optical density 1.8 to 600 nm using spectrophotometer.

A volume of 0.5 mL of each bacterial suspension resulting from a very precise cell growth phase was introduced into conical flasks containing the polyethylene fragments previously sterilized as indicated in conical flasks containing a sterile solution of either  $\text{CuSO}_4$  or  $\text{ZnSO}_4$ , of precise concentration, were stirred to homogenize the bacterial suspension in the water column, then incubated at laboratory temperature ( $25 \pm 2^\circ\text{C}$ ) for determined times which

were from 4, 12, 18, and 24h. The experiments were carried out partly in pure culture condition (solution containing a single bacterial species and in mixed culture condition (solution containing the 2 bacterial species at the same time). The controls are each time prepared in the absence of heavy metal salt, then incubated at the same time.

At the end of each incubation period, each fragment was then introduced into 10 mL of physiological water. The stalling of the adhered cells was carried out by vortexing at increasing speeds for 30 seconds, in 3 consecutive series of 10 mL of sterile physiological water for a total volume of 30 mL. This technique made it possible to obtain the maximum number of detached cells.

Bacteriological analysis was carried out on the stock suspensions, the suspensions resulting from the release of cells in the absence of heavy metal salts and the suspensions resulting from the release in the presence of heavy metal salts. A volume of 0.1 mL of the suspensions to be analyzed was inoculated by the surface spreading technique on selective media.

### Data analysis

The average values of the abundance of cultivable cells under each condition were been represented using histogram to show the evolution of adhered cell in the presence of heavy metal. Statistical analysis was performed using SPSS version 25.0. The correlation coefficients among considered parameters were assessed using Spearman correlation test. The H test from Kruskal-Wallis was performed in order to compare the abundances of *S. aureus* and *A. hydrophila* adhered to polyethylene in the presence of the different metal salts considering each experimental condition.

## Results and Discussion

### Bacterial growth curves

The evaluation of the growth of *S. aureus* and *A. hydrophila* bacteria in the non-renewed peptone medium describes a hyperbolic curve in 4 phases. When considered  $t_{0h}$  as the initial moment, the 4 growth phases were temporally divided as follow: the lag growth phase from  $t_{0h}$  to  $t_{3h}$  and  $t_{0h}$  to  $t_{2h}$ , the exponential growth phase from  $t_{3h}$  to  $t_{10h}$  and  $t_{2h}$  to  $t_{13h}$ , the stationary growth phase from  $t_{10h}$  to  $t_{21h}$  and  $t_{13h}$  to  $t_{21h}$ , and finally the decline growth phase from  $t_{21h}$  to  $t_{26h}$  and  $t_{21h}$  to  $t_{28h}$  respectively for *S. aureus* and *A. hydrophila*.

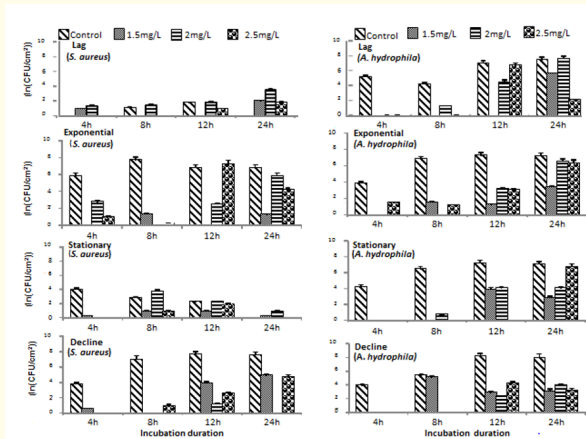
### Abundances of bacteria adhered to polyethylene in the presence of heavy metal salts in pure culture condition

In the presence of  $\text{CuSO}_4$ , the highest value of *S. aureus* coming from lag growth phase was recorded after 24 hours of incubation at a concentration of 2 mg/L of  $\text{CuSO}_4$ . As for *A. hydrophila*, the maximum value was 8 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) was noted after 24 h of incubation in the presence of 2 mg/L of  $\text{CuSO}_4$  (Figure 1). For cells coming from exponential growth phase, the highest abundances of *S. aureus* and *A. hydrophila* adhered to polyethylene reached values of 7 and 6 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) respectively. These values were recorded at a concentration of 2.5 mg/L of  $\text{CuSO}_4$  after 12 h and 24 h of incubation respectively for *S. aureus* and for *A. hydrophila*.

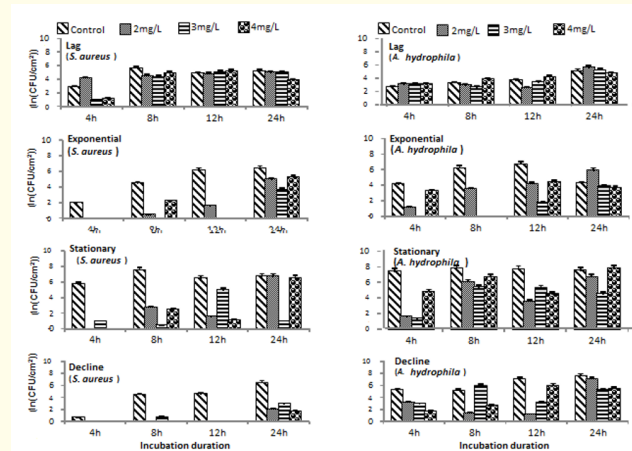
For cells harvested from stationary growth phase, the densities of adhered cells of *S. aureus* varied between 0 and 4 units ( $\ln(\text{CFU}/\text{cm}^2)$ ). The abundances of *A. hydrophila* oscillated between 0 and 7 units ( $\ln(\text{CFU}/\text{cm}^2)$ ). The maximum value of 7 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) was recorded at the concentration of 2.5 mg/L of  $\text{CuSO}_4$  after 24 hours of incubation (Figure 1). When cells came from decline growth phase, it was noted that the abundances of adhered cells were low. The value of 5 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) was recorded at the same time for *S. aureus* and for *A. hydrophila* at the concentrations of 2.5 mg/L and 1.5 mg/L after 24 hours and 8 hours of incubation respectively (Figure 1). However, the control tests carried out in the absence of  $\text{CuSO}_4$  revealed that the highest abundances of *S. aureus* cells adhered to the fragments reached 8 units ( $\ln(\text{CFU}/\text{cm}^2)$ ). The abundances of the adherent cells varied from 4 to 8 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) for *A. hydrophila*. The maximum abundances were recorded after 24 hours of incubation.

In the same condition, the abundances of *S. aureus* cells adhered to polyethylene reached 7 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) in the absence of  $\text{ZnSO}_4$  and the abundances of *A. hydrophila* cells adhered to the polyethylene oscillated between 3 and 8 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) (Figure 2). For cells coming from lag growth phase, the densities of the adhered *S. aureus* and *A. hydrophila* cells fluctuated between 1 and 5 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) and between 3 and 6 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) respectively in the presence of  $\text{ZnSO}_4$ . For cells harvested from the exponential growth phase, the highest abundances of adhered *S. aureus* cells reached 5 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) at concentrations of 2 and 4 mg/L of  $\text{ZnSO}_4$  after 24 hours of incubation and the highest cell densities of *A. hydrophila*, reached 6 units ( $\ln(\text{CFU}/$





**Figure 1:** Temporal evolution in pure culture of the abundances of *Staphylococcus aureus* and *Aeromonas hydrophila* cells adhered to polyethylene in the presence of  $\text{CuSO}_4$ ; bacteria cells harvested from Lag growth Phase, Exponential growth Phase, Stationary growth Phase, and Decline growth Phase.



**Figure 2:** Temporal evolution in pure culture of the abundances of *Staphylococcus aureus* and *Aeromonas hydrophila* cells adhered to polyethylene in the presence of  $\text{ZnSO}_4$ ; bacteria cells harvested from Lag growth Phase, Exponential growth Phase, Stationary growth Phase, and Decline growth Phase.

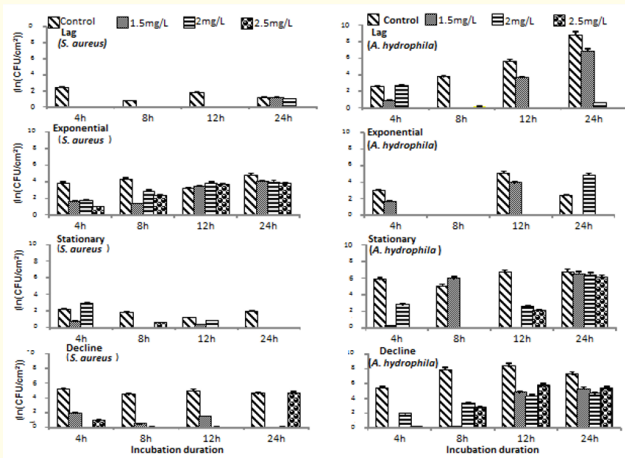
$\text{cm}^2$ ). This value was noted in the presence of 2 mg/L of  $\text{ZnSO}_4$  after 24 hours of incubation. The highest abundances of adhered *S. aureus* and *A. hydrophila* cells reached 7 and 8 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) respectively when cells harvested from stationary growth phase. When cells coming from decline growth phase, the abundances of *S. aureus* cells were very low and the abundances of *A. hydrophila* adhered reached 7 units ( $\ln(\text{CFU}/\text{cm}^2)$ ). It was observed that the abundances of *A. hydrophila* adhered increase with the incubation duration (Figure 2).

#### Abundances of bacteria adhered to polyethylene in the presence of heavy metal salts in mixed culture conditions

In general, the abundances of *A. hydrophila* and *S. aureus* adhered to polyethylene obtained in the absence of  $\text{CuSO}_4$  varied from 2 to 9 and from 1 to 5 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) respectively. Overall, these densities were higher than those obtained on fragments subjected to copper sulfate (Figure 3). In the presence of  $\text{CuSO}_4$ , the abundances of adhered *S. aureus* cells were very low when the cells were harvested from the lag growth phase. The abundances of adhered *A. hydrophila* reached 7 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) at a concentration of 1.5 mg/L after 24 h. It was noted that the cell abundance of *A.*

*hydrophila* adhered increase with incubation duration (Figure 3). From cells coming from the exponential growth phase, the densities of *S. aureus* cells adhered to polyethylene fluctuated between 1 and 4 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) and the abundances of adhered cells *A. hydrophila*, reached the maximum value of 5 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) at a concentration of 2 mg/L after 24 h (Figure 3). The abundances of adhered *S. aureus* cells were low when cells were coming from stationary growth phase. The abundances of *A. hydrophila* reached the value of 6 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) after 24 hours at all concentrations of  $\text{CuSO}_4$ . When cells were coming from decline growth phase, the densities of *S. aureus* cells reached 5 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) at a concentration of 2.5 mg/L after 24 h. The abundances of *A. hydrophila* for reaching the value of 6 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) at 2.5 mg/L after 12 hours of incubation (Figure 3).

The highest abundances of *S. aureus* and *A. hydrophila* adhered to polyethylene in the absence of  $\text{ZnSO}_4$  reached values of 6 and 10 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) respectively (Figure 4). In the presence of  $\text{ZnSO}_4$ , the highest abundance of *S. aureus* cells adhered in mixed culture was obtained at 2 mg/L concentration of heavy metals salts after 24 h when cells stemming from lag growth phase. In the same

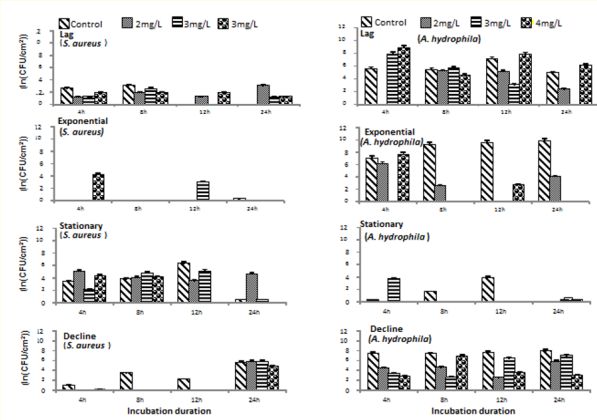


**Figure 3:** Temporal evolution in mixed culture of the abundances of *Staphylococcus aureus* and *Aeromonas hydrophila* cells adhered to polyethylene in the presence of  $\text{CuSO}_4$ ; bacteria cells harvested from Lag growth Phase, Exponential growth Phase, Stationary growth Phase, and Decline growth Phase.

condition, the abundances of *A. hydrophila* adhered reached the value of 9 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) with concentration of 4 mg/L after 4 h. For cells coming from exponential and stationary growth phase, the highest densities of *S. aureus* adhered cells sometimes are respectively 4 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) at a concentration of 4 mg/L and 5 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) at a concentration of 3 mg/L. The abundances of *A. hydrophila* reached the value of 8 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) and 4 units ( $\ln(\text{CFU}/\text{cm}^2)$ ) at 3 mg/L of  $\text{ZnSO}_4$  respectively. With cells harvested from decline growth Phase, the abundances of *S. aureus* adhered cells are low and the abundances of *A. hydrophila* adhered oscillated between 3 and 7 units ( $\ln(\text{CFU}/\text{cm}^2)$ ). The highest value in mixed culture was obtained after 24 hours with a concentration of 3 mg/L of  $\text{ZnSO}_4$  (Figure 4).

#### Relationships between considered parameters

The degrees of binding between the abundances of *S. aureus* and *A. hydrophila* adhered to polyethylene and the concentrations of copper sulphate were evaluated, the results are shown in table 2. In pure culture, a significant and negative association was recorded between the abundances of *S. aureus* and the different concentra-



**Figure 4:** Temporal evolution in mixed culture of the abundances of *Staphylococcus aureus* and *Aeromonas hydrophila* cells adhered to polyethylene in the presence of  $\text{ZnSO}_4$ ; bacteria cells harvested from Lag growth Phase, Exponential growth Phase, Stationary growth Phase, and Decline growth Phase.

tions of  $\text{CuSO}_4$  when cells coming from lag and stationary growth phases ( $P < 0.05$ ). This same result was observed with *A. hydrophila* with cells from exponential and stationary growth stages. In mixed culture, a very significant and negative association was noted between the abundances of *A. hydrophila* and concentrations  $\text{CuSO}_4$  when cells came from decline growth phase ( $p < 0.01$ ).

When the heavy metal salt is  $\text{ZnSO}_4$ , a significant associations between the abundances of bacteria adhered to polyethylene and the different concentrations were noted only in pure culture. It appears that a very significant and negative link is observed between the concentrations of this salt and the abundances of *S. aureus* for cells coming from stationary and decline growth phases ( $p < 0.05$ ). Likewise, the abundances of *A. hydrophila* decrease very significantly ( $p < 0.01$ ) with the increase of  $\text{ZnSO}_4$  concentration with cells from exponential growth phase. Studies also showed that the incubation duration influence significantly ( $p < 0.01$ ) the growth of both bacteria in each experimental condition.

Under pure culture, from one incubation period to another, there was an overall significant difference ( $P < 0.01$ ) between the mean abundances of *S. aureus* and *A. hydrophila* adhered in the

Bacterial species and cell growth phases		Experimental conditions and correlation coefficients			
		In the presence of CuSO <sub>4</sub>		In the presence of ZnSO <sub>4</sub>	
		Pure culture	Mixed culture	Pure culture	Mixed culture
<i>S. aureus</i>	Lag	-0.949*	0.544	0.600	0.500
	Exponential	-0.632	-0.800*	0.775	0.816
	Stationary	-0.943*	-0.312	-1,000**	0.200
	Decline	0.800	-0.272	-0.949*	0.400
<i>A. hydrophila</i>	Lag	0.400	0.211	0.800	-0.200
	Exponential	-0.994*	0.500	-1,000**	0.447
	Stationary	-0.312	0.400	0.048	0.056
	Decline	-0.272	-1,000**	-0.400	0.256

**Table 2:** Spearman “*r*” correlation coefficients between abundances of adhered bacteria and concentrations of heavy metal salts with respect to growth phases at each culture condition.

Number of observations: 36; \*:  $P < 0,05$ ; \*\*:  $P < 0.01$ .

presence of ZnSO<sub>4</sub> differ significantly (Table 3). From one growth phase to another, the abundances of *S. aureus* differed significantly in the presence of ZnSO<sub>4</sub> in pure culture as well as in mixed culture. The abundances of *A. hydrophila* adhered in the presence of CuSO<sub>4</sub> and ZnSO<sub>4</sub> differ significantly ( $p < 0.05$ ) for the case of CuSO<sub>4</sub> and significantly ( $p < 0.05$ ) for the case of ZnSO<sub>4</sub> from a growth phase to the another in mixed culture. No significant difference was noted with the abundances of *A. hydrophila* and the heavy metal salts from one growth phase to another in pure culture. In general, there is no significant difference in the abundances of the adherent bacterial cells in the presence of heavy metal salts from one concentration to another (Table 3).

The growth of *S. aureus* and *A. hydrophila* on liquid medium described a four-phase hyperbola: lag, exponential growth, stationary and decline. The four phases of the growth curve of these bacteria reflected physiological states characteristic of these phases. In the lag phase, the cells have zero-growth rate, they adapt to the new medium and synthesize enzymes necessary to metabolize the new substrates [15]. In the exponential growth phase, bacteria use the metabolites of the medium and actively duplicate. The stationary growth phase is characterized by a balance between the bacteria

Experimental conditions and parameters considered		Bacterial species and salt considered			
		<i>S. aureus</i>		<i>A. hydrophila</i>	
Pure culture	Heavy metal salts concentration	CuSO <sub>4</sub>	ZnSO <sub>4</sub>	CuSO <sub>4</sub>	ZnSO <sub>4</sub>
	Incubation duration	P = 0.008**	P = 0.001**	P = 0.000**	P = 0.001**
	Phase growth	P = 0.516	P = 0.001**	P = 0.898	P = 0.075
	Concentration	P = 0.725	P = 0.794	P = 0.805	P = 0.470
Mixed culture	Incubation duration	P = 0.667	P = 0.691	P = 0.031*	P = 0.637
	Phase growth	P = 0.000**	P = 0.003**	P = 0.020*	P = 0.001**
	Concentration	P = 0.519	P = 0.976	P = 0.245	P = 0.608

**Table 3:** Comparison amongst abundances of *S. aureus* and *A. hydrophila* adhered to polyethylene obtained at each incubation period, each growth phase, each concentration of heavy metal salts in pure and mixed culture.

\*:  $P < 0.05$ ; \*\*:  $P < 0.01$ .

which die and those which multiply. Finally, the phase of decline is the moment when we notice the depletion of reserves and an

accumulation of toxic substances. There is a decrease in viable organisms and cell lysis under the action of endogenous proteolytic



enzymes. However, growth persists by release of substances during lysis (cryptic growth) [16].

The results obtained also show that heavy metals have an influence on the adhesion of *S. aureus* and *A. hydrophila*. The increase in the abundance of adhered cells is linked to the decrease in the concentration of  $\text{CuSO}_4$  and  $\text{ZnSO}_4$ . The high adhesion rate recorded in *A. hydrophila* with both  $\text{CuSO}_4$  and  $\text{ZnSO}_4$  could be explained by the nature of the bacteria wall, their optimal growth pH and the presence of flagellation. Indeed, *A. hydrophila* is a Gram-negative bacterium having a polar flagellation [17]. The wall thickness of these bacteria being very low and *A. hydrophila* will therefore be more sensitive to the presence of salts in solution. The presence of the flagellum, a very useful appendix in adhesion, is also an asset allowing it to get closer to the support and to adhere to it. Unlike, *A. hydrophila*, *S. aureus* is gram positive and does not exhibit flagellation. These characteristics make it less susceptible to the presence of metal salts due to the considerable thickness of the wall and also a slow approach to the substrate due to the absence of flagellum. This difference in adhesion rate could also be explained by the variability of the pH and of the electrical conductivity induced by the concentrations of heavy metal salts.

Indeed the electrical conductivity is the parameter which reflects the degree of mineralization of the water; The dissolved ionic species, by their nature as well as their concentration, considerably affect the bioadhesion of microorganisms to supports [18]. The high values of conductivity would have no overall stress effect on bacteria; these observations corroborate those of Nola, *et al.* (2001, 2002) [19,20] who found that bacteria of the genus *Staphylococcus* and *Aeromonas* tolerate strong mineralization. However, for a concentration of  $2.5 \text{ mg.L}^{-1}$  of  $\text{CuSO}_4$ , the increase in the abundance of *A. hydrophila* adhered to polyethylene would be significantly related to the decrease in electrical conductivity. This could be explained by the fact that at this value of the  $\text{CuSO}_4$  concentration, the ionic species ( $\text{Cu}^{2+}$  and  $\text{SO}_4^{2-}$ ) have modified the surface properties of either the bacteria or the substrate and consequently increased the number of adhesion sites [2]. Likewise, the pH values indicate that overall there is only a significant link between the pH and the abundances of *S. aureus* adhering only to the  $2 \text{ mg.L}^{-1}$  value of  $\text{ZnSO}_4$ . The very weak correlations noted between the abundances of the adhered cells and the different pH values of the solutions of heavy metal salts obtained in the adhesion test could be

explained by the fact that these pH values belong to the growth pH range bacterial cells. Indeed, the growth pH of staphylococci varies between 4.0 and 9.3 with an optimum located around neutrality (pH 7 to 7.5) which also favors the production enzymes and toxins [21]. Furthermore, the low abundance of adhered bacterial cells can be explained by the toxic nature of heavy metals inducing the death of bacteria. Indeed, heavy metals such as copper have a biocidal effect and to a lesser extent play the role of stressors [22,23]. Test studies on the formation of biofilms with copper as a stressor have shown that copper prevents the formation of biofilms in certain bacteria [23]. Studies shown that the action of heavy metals on bacterial cells was characterized by the peroxidation of membrane lipids inducing the death of the latter [24].

In mixed culture, the abundances of bacteria adhered to polyethylene are lower than those obtained in pure culture. In this case, the abundances of *S. aureus* cells are lower in mixed culture than in pure culture. The fluctuations in the abundance of *S. aureus* and *A. hydrophila* in mixed culture could be explained by a phenomenon of competition between the two bacteria. In a hostile environment, *S. aureus* is able to secrete exotoxins [25]. These exotoxins allow the bacteria to fight against the stress caused by the simultaneous presence of heavy metal salts and *A. hydrophila*. The high abundances of *A. hydrophila* compared to those of *S. aureus* in mixed culture can be explained by its polar flagellation [17]. Thus, in response to the stress caused by the simultaneous presence of heavy metal salts and *S. aureus*, *A. hydrophila* will arrive more quickly at the level of the substrate to adhere to it more quickly than *S. aureus* which is a cocci without flagellation. The duration of incubation of the two bacterial strains in solutions of heavy metal salts also has a very great influence on the adhesion of these bacteria to the polyethylene. There is a clear significant difference ( $p < 0.001$ ) between the abundances of *S. aureus* and *A. hydrophila* and the incubation time in the different salt solutions. For a prolonged period, bacteria adhere better to the substrate. These results corroborate those of some authors who found that the abundances of *A. hydrophila* cells adhered to polyethylene increased with the duration of incubation [26]. This could be explained by the nature of the adhesion substrate. Indeed, some authors had observed that the cells of *A. hydrophila* adhered to organic substrate such as polybutethylene or polyethylene were more abundant for extended incubation time [27]. More *A. hydrophila* cells adhered to polythene at different

degrees with respect to incubation periods, cell surface hydrophobicity, cell growth phases and disinfectant concentrations in water [28]. Indeed, interactions exist between solid fragments that are invariant and bacterial cell surfaces which depend on the bacterial physiological condition [29]. Higher value of adsorption coefficients implies greater adsorption capacity and lower adsorption coefficient implies lower polythene fragments adsorption capacity. The variability sometimes observed in this substrate adsorption potential could be due to the variability of the number of adhesion sites groups on fragments [30,31]. Concerning particularly *S. aureus*, many studies shown that adhesion of this bacteria is influenced by wettability and average surface roughness [32]. Other Studies also reported preferential attachment of *S. aureus* on laser-induced periodic surface structures with a static water contact angle of 79° [33]. Furthermore, it was observed increases in *S. aureus* retention on superhydrophobic laser-treated titanium surfaces with large 10-20 µm features compared to polished samples [34]. However, it was observed reductions in *S. aureus* biofilm formation on laser-induced periodic surface structures and nano-pillars compared to polished titanium samples [35].

The abundances of *S. aureus* and *A. hydrophila* cells vary from one growth phase to another, especially in mixed culture. The growth phases in fact have a significant influence on the rate of adhesion of bacteria to polyethylene in the presence of heavy metal salts; the highest abundance values, 8 ln units (CFU/cm<sup>2</sup>) and 9 ln units (CFU/cm<sup>2</sup>), were obtained respectively in the exponential growth phase and in the lag phase. Overall, it was noted that whatever the growth phase, there is a significant difference ( $p < 0.05$ ) between the abundances of *S. aureus* in the presence of ZnSO<sub>4</sub> in monoculture and polyculture on the one hand and 'on the other hand, concerning *A. hydrophila* this difference is noted only in polyculture. The physiological state of the bacteria strongly influenced the speed of adhesion, due to the accession rate that varies depending on cellular growth phase that bacteria come from [11,36]. During the adsorption test, the exponential growth phase results in a high cell activity whereas the stationary growth phase has a slowing down of this activity, resulting in chemical modifications to the surface of the cell [11,36]. Some authors noticed that, the physiology of bacteria changed at each stage of growth [37,38]. Therefore, the fluctuations in the abundances of the adhered bacteria could be explained by the variation in the physiological state of the bacteria *S. aureus* and *A. hydrophila*.

## Conclusion

The bacteria *S. aureus* and *A. hydrophila* have the ability to adhere to polyethylene in the presence of CuSO<sub>4</sub> and ZnSO<sub>4</sub>. The growth phases, the incubation period and the culture conditions are factors which vary this adhesion. Low concentrations of heavy metals seem to favor relatively high number of adhered cells. The increase in the contact time between bacteria and heavy metal solutions is significantly related to an increase in the adhesion rate. Maximum abundances were recorded after 24 hours of incubation. The temporal evolution of the pH and the electrical conductivity of solutions of metal salts indicates that these parameters have little influence on the adhesion of bacteria. The fight against the presence of *S. aureus* and *A. hydrophila* bacteria in water could be achieved by the use of heavy metal salts. In fact, in the laboratories manufacturing adsorbents used in wastewater treatment plants, the use of CuSO<sub>4</sub> and ZnSO<sub>4</sub> is recommended in order to improve the adsorption capacities.

## Conflict of Interest

The authors declare that they have no competing interests.

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