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Survival of Bacterial Pathogen Isolated from Urine in Surface Water in Sebou River Estuary (Morocco)

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ABSTRACT

Hospital effluents are one of the main sources of contamination of groundwater and rivers if they are collected by urban networks and sent to wastewater treatment plants (WWTP), without prior treatment. These effluents are potential vectors of resistant pathogenic bacteria and could contribute to the spread of these strains in the environment. The Sebou River is used as a receptacle for domestic water treatment from the city of Kenitra. The main of the study is to analyze the effect of pH (8.2, 7.5, and 6.5) at a temperature of 22°C on the behavior of three pathogenic bacteria isolated from urine in filtered and sterilized water Sebou River. The water of Sebou River was inoculated with the three bacterial strains tested by a concentration that varied from 10^6–10^8 CFU/mL, then incubated for 30 days. The results of the obtained analyses showed that the pH affects the survival of the tested bacterial strains. For some strains, the alkaline pH at 22°C is a beneficial effect that prolongs survival. The main finding from the study was that the three strains of *Escherichia coli* at a pH of 8.2 have a better survival that reaches the 7th day and then declines. On the other hand, with a strain of *Klebsiella pneumoni* and *Staphylococcus aureus* at pH 7.5, the action effects are harmful; it limits survival to 72 hours and causes a rapid decrease in the number of bacteria. We have shown in our experiments that bacteria survive much better at low temperatures and basic pH.

Keywords: survival; bacterial pathogens; water pollution; wastewater; pH; temperature; Sebou River.

INTRODUCTION

Water, the source of life, is essential for all living beings. This natural wealth is more remarkable in all countries with arid or semi-arid climates that suffer from water deficits and irregularities in the water supply over time and space. Indeed, water has a fundamental role in many fields such as drinking water treatment, agriculture, industry, health care institutions, electricity production as well as domestic uses (Hamdy et al., 2003). According to studies carried out by the United Nations (UN), almost half of the population of the Mediterranean countries will be in a situation of water scarcity or shortage in 2025(Chartier et al., 2014). Morocco is currently experiencing water scarcity (less than 1000 $m^3.inhab^{-1}.year^{-1}$) and is expected to experience water scarcity (less than 500 $m^3.inhab^{-1}.year^{-1}$) after 2025(Tekken and Kropp, 2012). Climate change could exacerbate the negative impacts of scarcity, Spatio-temporal disparity, and severe degradation that characterize water resources in this semi-arid region. Indeed, with the demographic increases, freshwater resources are exposed to various pollutions of multiple origins: industrial, urban, and agricultural, generating damage to man and his environment (DeNicola et al., 2015). This pollution generates numerous waterborne diseases which can be the cause of certain epidemics(Ravindra et al., 2019). The city of Kenitra, like other cities in the country, has not spared the scourge of all forms of pollution of water resources. Indeed, this city enjoys a strategic geographical position at the coastal outlet of the Gharb plain and the Sebou River, rich agricultural land(Behnassi et al., 2018). Similarly, it has a considerable environmental heritage, which is potentially an undeniable asset for its socio-economic development as well as many natural sites playing a leading role in the conservation of biodiversity(Azidane et al., 2018). With 431,282 inhabitants (GCPH, 2014), the city of Kenitra faces a situation of spontaneous urbanization and is under increasing pressure from rural immigration. Indeed, human activity leads to the production of wastewater discharged daily into nature. The overall volume of wastewater produced by the mentioned city has increased from 69,000 m^3/d in 2015 to 82,000 m^3/d in 2020. With the foreseeable population growth, this volume would attract 109,000 m^3/d by 2030(RAK, 2020). Healthcare establishments generate large volumes of liquid effluents that contain specific substances (drug residues, chemical reagents, disinfectants, detergents, X-ray developers and fixers) (Mackull'ak et al., 2021) and above all, are likely to disseminate pathogenic germs. Domestic and hospital wastewater are collected and transported to a municipal wastewater treatment plant (WWTP) where it undergoes several types of treatment to combat their harmful effects (Karami et al., 2021). However, although many micropollutants are eliminated by adsorption or biodegradation in the WWTPs, some molecules (drugs, fats, etc.) that are difficult to biodegrade pass through the treatment plants and are directly discharged into the Sbou River (Rout et al., 2021). In addition, due to the sometimes-intensive use of antibiotics in hospitals, certain bacterial strains can develop multiresistant antibiotics(Vincenti et al., 2014). The danger of pollution can therefore be accentuated by the presence of these germs in the public sewer system(Baquero et al., 2008). The fate of these discharges in the receiving environment may be of sanitary importance since the irrigation activity is threatened by bacterial contamination of fecal origin and chemical substances(Jaramillo and Restrepo, 2017). The present work is interested in studying the water quality by comparing the survival of three pathogenic bacteria at different pH in the water of Sebou River. The strains studied are of sanitary interest, *Escherichia coli,* and *Staphylococcus aureues* as control of fecal contamination and *Klebsiella pneumoniae* as a true pathogen for humans.

MATERIAL AND METHODS

Source of bacteria strain

In the current study, we have used several bacterial strains from different samples of medical origin. We have studied 12 strains (4 strains per bacteria): *Escherichia Coli, Klebsiella pneumoniae (K.pneumoniae), and Staphylococcus aureus (S. aureus)*. These strains that have been used are provided by the microbiology department of the medical analysis laboratories and the hygiene service of the El Idrissi Hospital. In addition, these strains are isolated from the urine of sick people. The identification of these 20 bacterial strains was reconfirmed by the use of conventional tests (gram staining, mobility, oxidase, and catalase) coupled with the use of the system of API 20E, 20 NE, and API Staph) and following standard procedures.

The Source of Sebou Rive

Physicochemical analyzes of Sebou River

The surface water was collected from the basin of Sebou River. The Lower Sebou estuary, crossing the city of Kenitra and drains a basin of 40,000 km^2, is one of the largest important basins in the Kingdom of Morocco and currently has a total population (of 49% of which are urban and 51% are rural). After collection, the water of Sebou River, was decontaminated by passing through a membrane filter (0.45 µm Millipore), sterilized for 20 min at 120°C, and used in microcosm experiments.

The Physico-chemical parameters are analyzed according to the protocols recommended by Rodier and al. (Rodier, 2009): pH, temperature, electrical conductivity NM ISO 7888, Biochemical Oxygen Demand (BOD5) WTW-OXYTOP, Chemical Oxygen Demand (COD) ISO 15705, Suspended Solids (SS) NM EN 872. In this study, the pH of the water of Sebou River is found to be neutral to slightly alkaline (Table 1).

Table 1. Characteristics physicochemical of water Sebou River used for the preparation of the inoculum

Temperature (T)	pH	Electrical conductivity (EC)	Suspended matter (SPM)	Chemical oxygen demand (COD)	Biological oxygen demand (BOD_5)
22.5 ± 3.5	7.5±1	9128± 6	491±48.5	814± 9.05	477±45.5

Preparation of inoculums

The bacterial strains were cultivated for 16 h at 37°C in trypticase-soy broth, and then 1 mL was transferred to 19 mL of boiling water for 4 h at 37°C, to obtain bacteria at the end of their exponential growth phase. The broth is then centrifuged (20 min at 3500 rpm), and the pellet is washed with physiological water (9% NaCI). The operation is repeated twice. The final cell pellet was diluted by buffered peptone water to give a final constituting the inoculum used to inoculate the microcosms cell density of approximately 10^6 colony-forming units (CFU/mL).

Preparation of microcosms

Microcosms included three series of three Erlenmeyer flasks (1 Erlenmeyer flask for each tested bacterial strain) of a capacity of 250 ml containing 100 mL of the same water sample from Sebou River at pH 7.5, 6.5 and 8.2. Each series is inoculated at the same time with a suspension of *Escherichia coli*, *Klebsiella pneumoniae*, and *staphylococcus aureus* (The final concentration of four strains in each Erlenmeyer flask varies from 1 to 3.10^6 CFU/mL). The temporal evolutions of the abundances of four tested bacterial strains are followed with a time step of 24 hours during 30 days by an indirect counting on a culture medium (nutrient agar).

Collection of samples

The culturable counts of bacterial strains in the water of Sebou River, in association with different pH, were observed as long as they were culturable and detectable by reading the number of colony-forming units (CFU). A sampling of four bacterial strains from flasks study of the water of Sebou River started after 30 min, which was considered as a 0 h reading. Subsequently, sampling was carried out after 30 minutes, 3, 7 h, and then at different day intervals until the bacteria was no longer culturable and detectable.

STATISTICAL ANALYSIS

Each experiment was performed three separate times, and standard deviations were calculated from the three replications. Differences in the data were considered statistically significant at the 95% level of confidence.

RESULTS AND DISCUSSION

Survival of *Escherichia coli*

At a pH 6.5, there is a decrease at different rhythms of the abundances of four strains. This decrease in the abundance can be explained by the depletion of the incubation medium, except for the E.coli 2 strain, which survives until the 7th day (Fig. 1a). At a pH of 7.5, a slight decrease in the abundance of four strains is remarked. It is noted that, beyond 72 hours, presence of the bacterial species is no longer detectable in the reaction medium. While the *E.coli 2* strain can survive longer than the other strains. From these 2 curves, it can be concluded that for T=22°C and whatever the studied pH is the survival of certain strains of *Escherichia coli* like the *E.coli 2* strain can survive longer than the other strains (Fig 1b). The evolution of the bacterial abundances at buffered pH 8.2 shows a slight and weak decrease in the number of bacteria. We remark that at pH 8.2, the number of viable cells remains relatively constant for a long time; since the optimal growth of *Escherichia coli* strains occurs at a pH that varies from 8 to 8.2. It is remarked that from 72 hours, the four strains of *Escherichia coli* are no longer cultivable in the of *Escherichia coli* survived much better than other conditions (Fig. 1c). The *Escherichia coli* (also called colibacil) is a radio-resistant bacterium, it is a common host of the commensal and intestinal microflora of the digestive tract of humans and animals. Several factors contributing to the elimination of E.coli in the aquatic environment are mentioned in works relating to the survival of these bacteria such as pH, temperature, nutrient availability, and nutritional level (Jang et al., 2017). During this study, the results show that the four strains of *Escherichia coli* under different pH conditions lose their cultivability after 72 hours (Figure 1). While certain strains resist these conditions to ensure their survival until the 7th day. However, the results are highly variable as a function of temperature and pH. Moreover, for T = 22 °C and pH 6.5, we have excellent survival, so the number of viable cells remains relatively constant for up to 72 hours; then all these strains will decline after the 7th day. Similarly (Liu et al. 2009; Pawlowski et al. 2011) have demonstrated that the survival of *Escherichia coli* seeded in filtered and autoclaved water was better at a low-temperature value. At

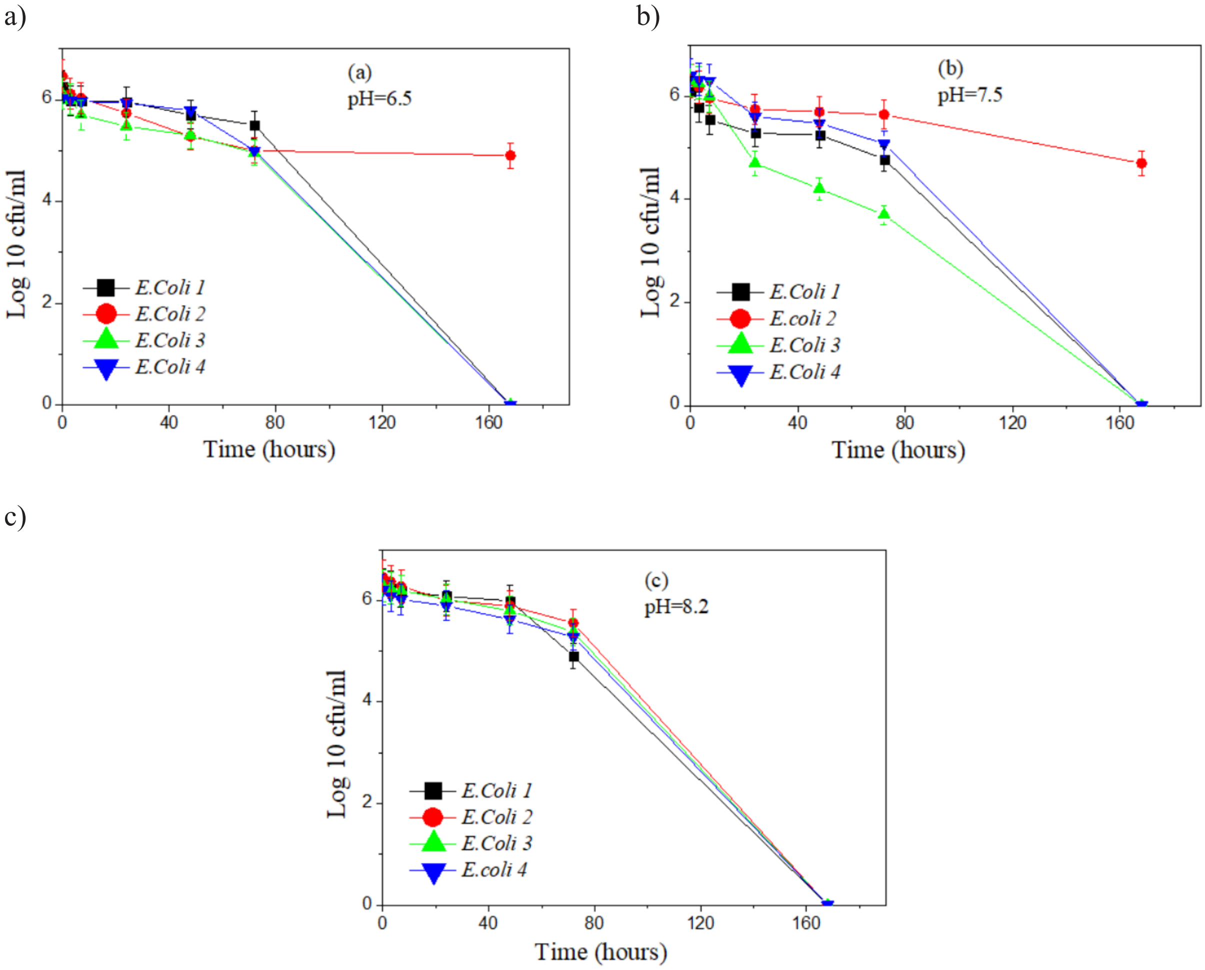

Figure 1. Survival of *Escherichia Coli (E.coli)* in surface water of Sebou River a pH (pH 6.5 (a), pH 7.5 (b) and pH 8.2 (c)) incubated at 22°C during 30 days

T = 22°C and after 7 days, all strains are no longer detectable in the reaction medium. This result agrees with those of many authors who have shown that low temperatures prolong the survival of *Escherichia coli* and high temperatures disadvantage and limit its survival (Franz et al., 2014; Farhangi et al., 2013)

Survival of *Staphylococcus aureus*

The medical strain *Staphylococcus aureu* strain not survive well in the surface water of Sebou River at pH = 6.5 and 7.5 (Fig. 2a and b), The curves of the evolutions of the abundances show almost the same shapes and are characterized by a slow decrease in their abundance. After 48 hours, the number of cultivable bacteria of the contact being very little different from that observed at $t = 0$. Between 48 and 72 hours, no tested strain was detected in the reaction medium; they underwent their decline at 7 days. In contrast the *Staphylococcus aureu* strain survived well in the surface water of Sebou River at pH-naturel 8.2 (Fig. 2c incubated at 22°C; this basic pH gives an identical effect for all strains (Figure 2). This effect is beneficial and gives a better survival of the strains, as well as the number of viable cells remaining relatively constant at the beginning and at the end of the experiment. *Staphylococcus aureus* is the most dangerous of all common staph bacteria. Their symbiotic life has been linked to the virulence of certain species, explaining that this bacteria is a major cause of the infection. The pH is also a parameter forming part of the explanatory models of the evolutions of the bacterial abundances in the Sebou River water. During our study, the results show that the low temperatures promote the survival of *Staphylococcus aureus* in water of River Sebou. These results are in agreement with those of (Levin-Edens et al., 2011; Topić et al., 2021). The authors have found that these bacteria survive best in water. This can be explained by the fact that low temperatures limit the energy expenditure of the bacterial cell

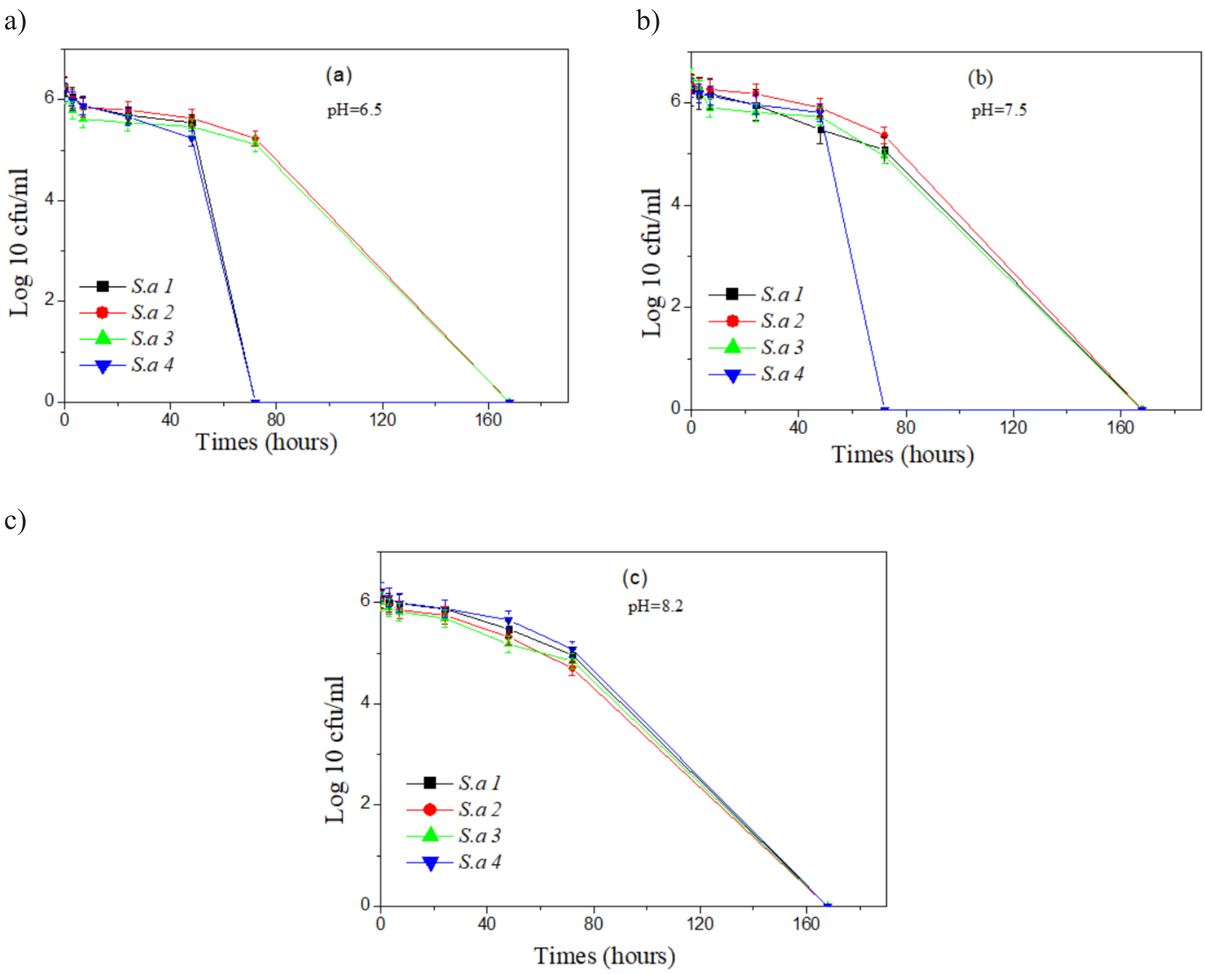

Figure 2. Survival of *Staphylococcus aureus* (S.a) in surface water of Sebou River at a pH (pH 6.5 (a), pH 7.5 (b) pH 8.2 (c)) incubated at 22°C during 30 days

by decreasing its metabolic activity. Indeed, in aquatic environments with high concentrations, bacterial growth can occur if the temperature is close to that which is favorable for optimal bacterial growth (Valero et al., 2009). Conversely, when the nutrients are lacking, survival is then favored by low temperatures which limit the energy expenditure of the bacterial cell by reducing its metabolic activity (Gaca et al., 2013)

Survival of *Klebsiella pneumoniae*

At different conditions of pH at temperature 22°C, the evolutions of the abundances of four strains of *Klebsiella pneumoniae* almost present the same shapes and are characterized by a slow decrease in their abundance. When the bacteria is incubated at pH 7.5, 6.5 and for T = 22°C (Fig. 3a), the curves are comparable and do not show a slight difference. Therefore,the cells lose their cultivarability. Firstly there is a rapid death for 2 days with a marked decrease in the number of bacteria, followed by a phase during which the number of viable cells tends to stabilize and is not dependent on the storage time. On the other hand, at pH of 8.2 incubated at 22°C, the same kinetics is observed with a constant number of viable cells at the end of the experiment. It can be seen that the survival of the *K.pneumoniae 1* strain is excellent throughout the duration of the experience (independent of the temperature and pH). This strain survives until the 7th day and then it has had its declines. It is also seen that, beyond 72 hours, the three other strains are no longer detectable in the reaction medium whatever the studied pH. In the same reaction medium and with the same value of pH 7.5, the curves have the same shape. So, it can be concluded that the pH 7.5 and 8.5 incubated at 22°C of the surface water of Sebou River contains all the necessary elements that can facilitate the survival of *Klebsiella pneumoniae* (Fig. 3b and c). The results present evolutions of the abundances almost with the same shapes and a weak difference (Kalaiselvi et al., 2016; Moghtaderi et

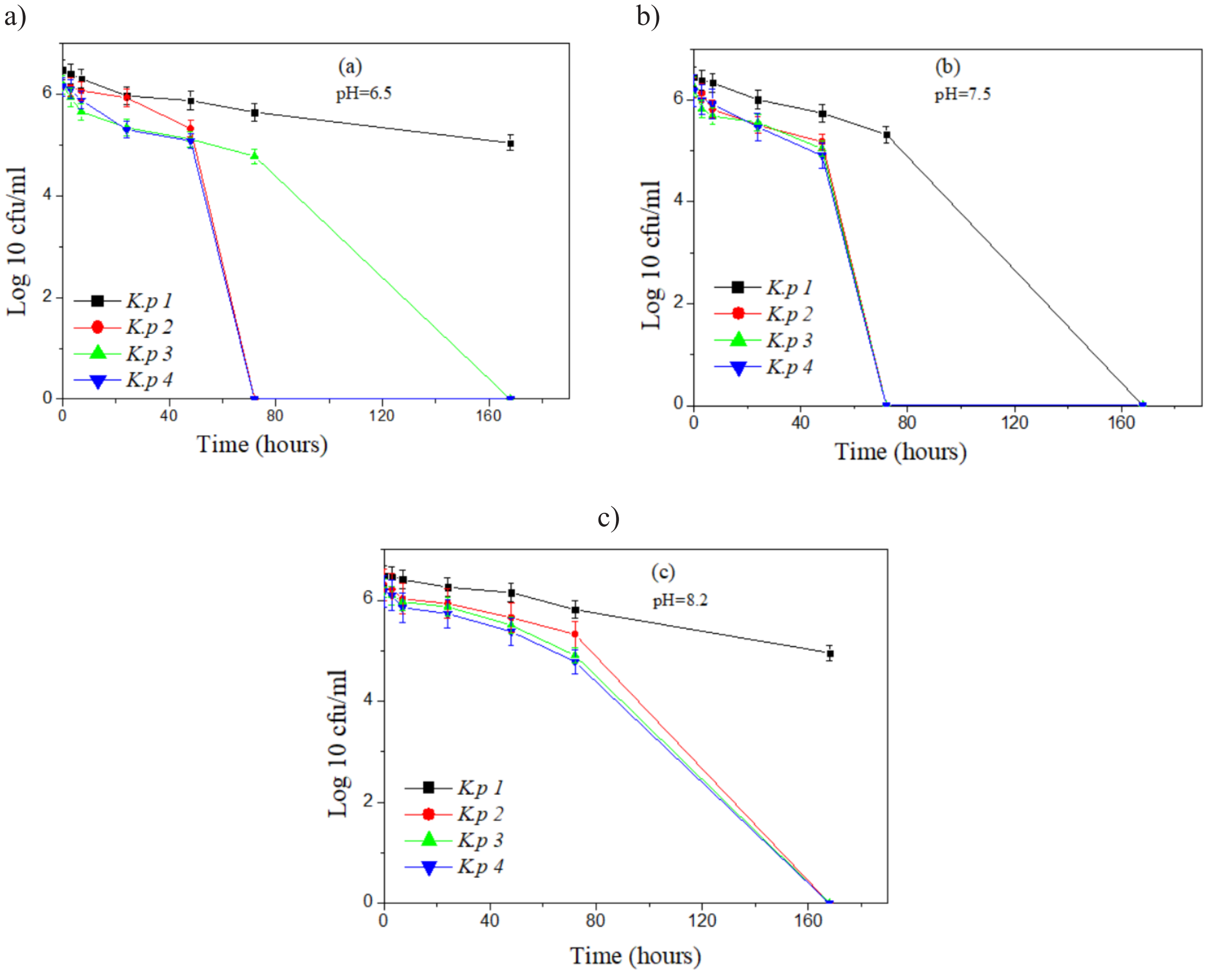

Figure 3. Survival of *Klebsiella pneumonia (K.p)* in surface water of River Sebou at a pH (pH 6.5 (a), pH 7.5 (b) pH 8.2(c)) incubated at 22°C during 30 days

al., 2021; Wadowsky et al., 1985). At different values of pH, the number of viable cells remains constant throughout the experience and there is even a small and slight decrease in the number of cultivable bacteria. Therefore, we can conclude that the natural pH of the water of Sebou River contains nutrients that may facilitate the survival of *Klebsiella pneumoniae*. This result is for with those of many authors: (Cao et al., 2015; Paczosa and Mecsas, 2016; King et al., 1988).

CONCLUSIONS

In the present study, survival is influenced by a number of environmental factors, in particular the original biotope of the strain, the nature of the survival medium (receiving medium), pH, and temperature. For the studying the different pH at temperature 22 °C, Enterobacteriaceae including *Escherichia coli* and *Klebsiella pneumoniae* will remain for some time in the waters of the River Sebou valley. During this stay, they will be able to adapt to a different living condition (decrease in temperature, supply of less assimilable nutrients). The present study also confirms in the low temperatures promote the survival of Staphylococcus aureus in a filtered and autoclaved water of River Sebou. The results show that the increase in the alkaline pH promotes and prolongs the survival of these strains and there is a slight decrease in the number of viable cells throughout the duration of the experience.

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