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New Alternative of Enhancing Hospital Hygiene Facing *Pseudomonas aeruginosa* Drug Resistance Impact of Hypertonic Saline Solutions on the Behavior of *P. aeruginosa*

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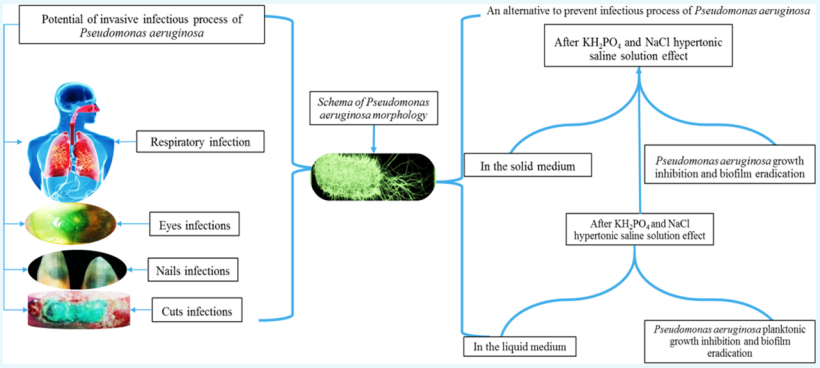
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Supporting Information



ABSTRACT: The objective of this study was to elucidate the antibacterial effect of hypertonic saline of KH_2PO_4 and NaCl solutions for enhancing hospital hygiene facing drug resistance of the biofilm of *Pseudomonas aeruginosa* isolated from a hospital environment on an agar cetrimide medium. The variations of *P. aeruginosa* biofilm density were measured following optical surface density by a correlated imaging method. Inhibition tests of 90% of the biofilm in a selective liquid medium monitored by spectrophotometry showed inhibition rates of 91.2 and 91.1% for minimal biofilm inhibition concentrations of 5.4 and 3.6% for NaCl and KH_2PO_4, respectively. The results of the eradication tests of 50% of the biofilm showed minimal biofilm eradication concentrations of 6.3 and 3.6% and eradication ratios of 50.6 and 50.9% for NaCl and KH_2PO_4, respectively. In the case of a selective solid medium, the results exhibited a minimal biofilm inhibition concentration of 4.2 and 3.9%, a biofilm inhibition rate of 90.8 and 90.5%, and a minimal biofilm eradication concentration of 2.7 and 3.3% for a biofilm killing quotient of 55.0 and 61.2% in NaCl and KH_2PO_4 cases, respectively. Accordingly, the results obtained demonstrated the potential of the hypertonic saline solutions and suggested that the employment of these solutions in hospital hygiene can be an alternative of prevention of nosocomial infections by *P. aeruginosa*.

1. INTRODUCTION

Pseudomonas aeruginosa, with *Staphylococcus aureus*, is one of the most common bacteria causing respiratory and nosocomial infections. In European intensive care units, the prevalence of these bacteria is approximately 4%, and its attributable mortality is around 13.5%, even with adequate antibiotic treatment.[1] In the multidrug resistance strains, the mortality rises up to 35.8% and the presence of multidrug resistance strains is identified as an independent predictor of hospital death.[2,3] In case of attack, the organism can be involved in the respiratory urinary, wound, and blood stream infections, mainly in patients with severe underlying diseases or impaired immune defense.[4]

P. aeruginosa resistant to diverse antibiotics appears as a potentially dangerous bacterium for the patients suffering from cystic fibrosis and comorbidities and in immunosuppressed patients and acquired immune deficiency syndrome sufferers. Those admitted in intensive care or the cancer patients for

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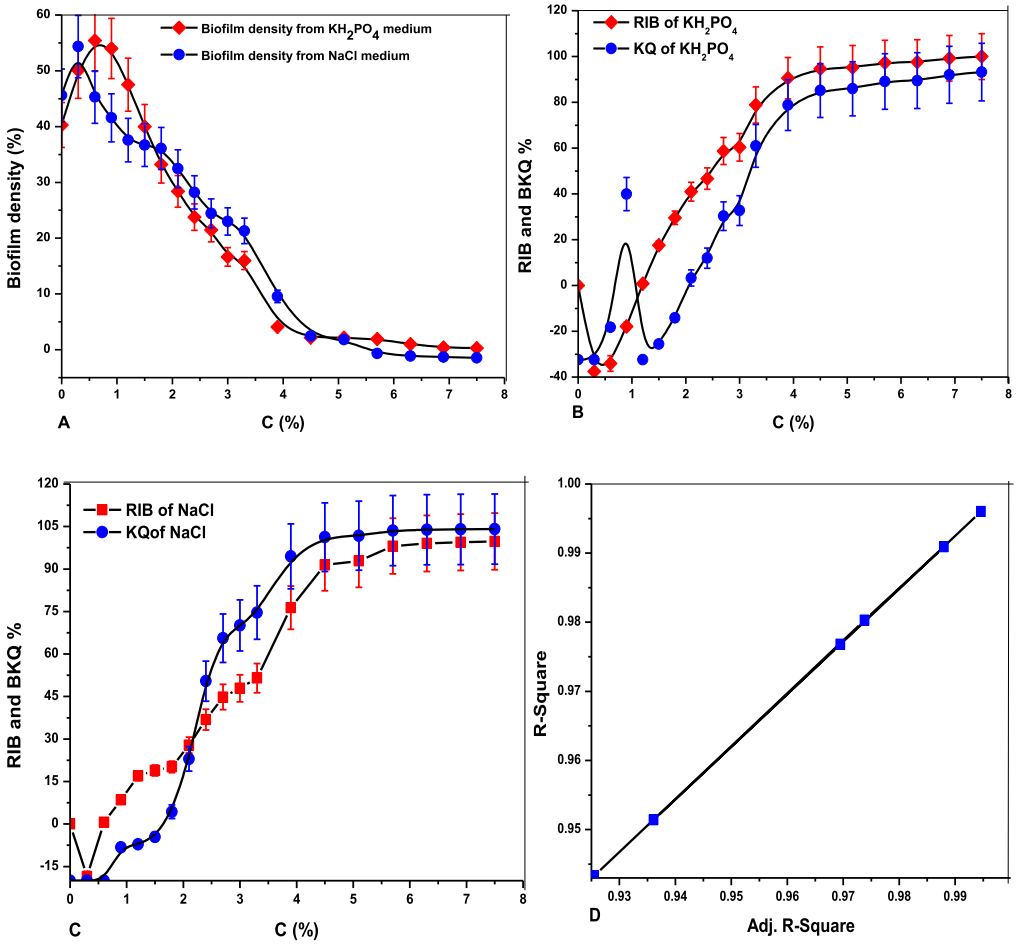


Figure 1. (A) Biofilm density of *P. aeruginosa* (KH_2PO_4 and NaCl). (B) RIB and BKQ of KH_2PO_4. (C) RIB and BKQ of NaCl in a solid "cetrimide agar" medium against the concentration *C* (%) and (D) correlation R^2 according to Adj. R^2 of all assays for 48 h of incubation, $T = 37$ °C, $P < 0.05$.

whom nosocomial infections establish the source of worsening of their health.[1,5,6] This resistance is also due to various factors secreted by *P. aeruginosa* such as phospholipase C, siderophores, alginate, exogenous toxin A, quorum sensing, and rhamnolipids, which facilitate its membership in the epithelial cells of patients.[6] The survival capacity in rudimentary conditions, natural resistance, and big diversity of plasmids of this bacterium confer a large potential of resistance acquisition. *P. aeruginosa* is the most known, most widespread species and the most pathogenic kind of *Pseudomonas*.[7,8]

These characteristics make an agent of nosocomial infections of preference particularly to the debilitated subjects hospitalized in intensive care.[9] The exogenous toxin A is an extracellular metabolite produced by *P. aeruginosa*, which participates actively in the process of invasion characterizing the most known process of infection.[10] The biofilm training of *P. aeruginosa* establishes a form of resistance, which makes the treatment of infections very difficult.[11] The main characteristic of *P. aeruginosa* virulence stays in its capacity of colonization of solid surfaces by the training of a biofilm in four main stages.[12] These biofilms have to part their physiological and genomic characteristics as well as of their quantitative importance in the ecosystems, establishing an essential interface, physiologically active, in nosocomial infections and the contamination of faucets hospitals waters.[7,11,12] The bacterial membership in the host cells is generally the first stage of the infectious process.[6] It can be associated with fimbriae and free toxin or found in solution.[13,14] The quorum sensing regulates this training of biofilm, a molecular descriptive mechanism that control the growth conditions of bacteria in a given environment.[1] The chromosome of *P. aeruginosa* possesses a genomic code in most particular of pathogenesis factors and multiple proteins conferring its resistance to various classes of antibiotics.[15] The size, complexity, and variability of its genome reflect an adaptive evolution of the species allowing its survival in various environments. Indeed, the photogrammetry is largely used for two-dimensional size estimation and pattern recognition applications in biology. Photogrammetry became a viable, practical, and accurate tool for the noninvasive assessment of biological systems and their physical environment.

Specifically, the accuracy of noninvasive body mass estimated for pinnipeds based on photogrammetrically derived body volume assessments has been determined. In addition, Institute Hatfield Marine Science[23] have tested if the body conditions can be estimated from a combination of the photogrammetrically derived and the morphometric measurements, specifically how accurate are standard morphometric measurements of vertebrates that are derived from photogrammetric measurements. Also, in their study, Kim et al.[24] and Dalamagkidis et al.[25] used the smartphone photogrammetry that can be operated in a 3G network environment at any time or location. This technology combines aerial and terrestrial photogrammetry and is commonly applied over short distances. Contrary to the conventional aerial photo-

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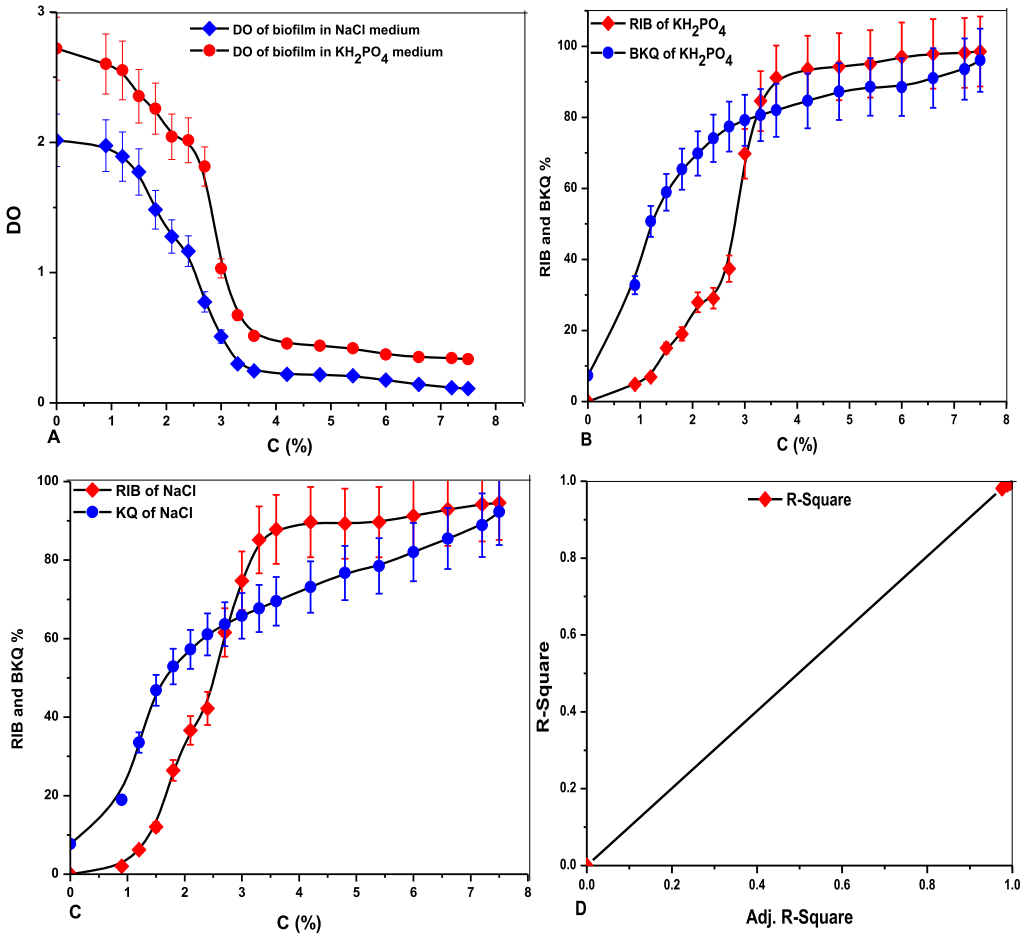


Figure 2. (A) Biofilm density of *P. aeruginosa*, (B) RIB and BKQ of KH_2PO_4, (C) RIB and BKQ of NaCl in a liquid cetrimide medium against the concentration and (D) correlation R^2 vs Adj. R^2 for 48 h of incubation at T = 37 °C, $P < 0.05$.

grammetry, such a system can be operated at a relatively low cost and applied on a real-time basis.[26] Tyson et al.[27] developed an advanced full-field imaging method using image correlation photogrammetry, used for measuring the variations in real biological mechanical systems such as bones, tendons, ligaments, and even tissues such as blood vessels. Since the work of Cocito et al.,[28] the quantitative estimation of the total mass of an organism measured as volume or mass (live, dead, dry, or ash-free weight) was considered of fundamental and practical importance in benthic ecology, in particular for the understanding of the energy flow, cycling of organic matter, carbonate production, and exploitation of demersal fish stocks in aquatic ecosystems.

The main purpose of this study was to characterize the effects of potassium dihydrogen phosphate (KH_2PO_4) and sodium chloride (NaCl) on *P. aeruginosa* biofilm density stemming from the hospital environment. Specifically, this study is considered to elucidate the potential of the hypertonic solution for an eventual use in hospital hygiene solutions. The results were interpreted according to the minimum biofilm inhibitory concentration (MBIC) corresponding to the biofilm inhibition rate (RIB) and minimum biofilm eradication concentration (MBEC) corresponding to the biofilm killing quotient (BKQ). The applied focal photogrammetric method, minimal inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) by the successive dilutions technical and the physiological behavior of this microorganism in the medium were also considered.

2. RESULTS AND DISCUSSION

The estimation results of the biofilm density on 52 Petri dishes were expressed in the percentage of the area occupied by the biofilm [Figure 1A] and correlated by statistical analysis [Figure 1D]. In the range of [0.3−0.9]%, low concentrations of KH_2PO_4 stimulated the biofilm growth in a solid medium and favored membrane adhesion of *Pseudomonas* by cell−cell, cell−surface, and biofilm−surface interactions. This growth was accompanied by a particulate destruction, characteristic of mutant cells lacking resistance genes during duplication, as well as the destruction of highly stressed cells. The latest were in a vegetative state (Figure 1B), respectively, at 0.6% with BKQ = 9.26 and 0.9% with BKQ = 47.43%.

However, NaCl stimulates growth only from 0.3% without destroying the biofilm. The results showed that inhibition by KH_2PO_4 began from a concentration of 1.2% against 0.6% for NaCl (Figure 1B,C). The results obtained by the agar diffusion method demonstrated the development of a very weak zone of inhibition with the disks impregnated with NaCl, which gave a diameter of 1.2 cm, hence the effect was bactericidal. Disks impregnated with 10% of KH_2PO_4 provoked the formation of three zones of different diameters according to the diffusion concentration gradient during 24 h of incubation at 37 °C. The diameters obtained by KH_2PO_4 were of 1.2, 3.5, and 4.2 cm.

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Figure 2 shows the optical density of the biofilm against the hypertonic saline concentrations after 48 h of incubation at 37 °C. The results obtained showed a zone of low resistance of *P. aeruginosa* in the concentration range of [0.3−1.5%] where less than 20% of the biofilm was inhibited with RIB of 15.08 and 12.05% and with BKQ of 35.17% and 25.44% for KH_2PO_4 and NaCl, respectively (Figure 2B,C). Figure 2D shows the relative correlation of the optical density of the biofilm as a function of the hypertonic saline solutions with $R^2 > 0.9$ for all the tests carried out. The results obtained by the successive dilution method showed an MIC of 5.1% for NaCl. In contrast, the bacterial cells inhibited in a solid medium allowed to determine the MBC of NaCl of 6.6%. In addition, the results obtained with KH_2PO_4 demonstrated an MIC of 5.7% and an MBC of 6.6% after 24 h of culture at 37 °C. This slight increase in the MIC of KH_2PO_4 was the consequence from its stimulating effect on the *P. aeruginosa* strain during its growth in test tubes with a concentration lower than 5.7%.

According to the Burt work, the MIC is defined as 90% and the minimum concentration eradication of a biofilm is 50% in a selective environment of the physiological conditions of *P. aeruginosa* during an incubation time of 48 h as demonstrated in Figures 1A and 2A. Indeed, in the two selective medium tests, the action of KH_2PO_4 showed a particularly powerful effect in the eradication of the biofilm by cellular degeneration as a reverse effect of the growth stimulation in the range of 0.3−0.9%. The effect of KH_2PO_4 constitutes a wide difference in action between the two salts in both environments. The microscopic examination of colonies showed that in a solid environment, KH_2PO_4 provoked an intense secretion of mucoids in the range of 1.2−3.3% (Figure 3).

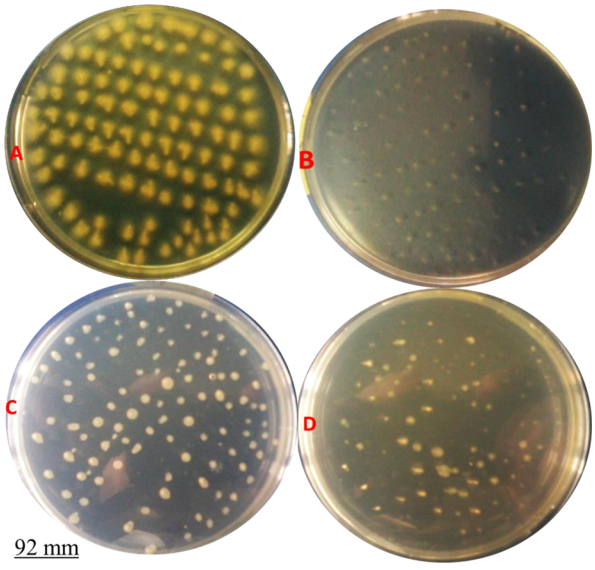


Figure 3. (A) *P. aeruginosa* live in 1.2% of KH_2PO_4, (B) *P. aeruginosa* dead in 3.6% of KH_2PO_4, (C) *P. aeruginosa* live in 1.2% of NaCl, and (D) *P. aeruginosa* dead in 3.6% of NaCl.

This secretion is characterized by the overproduction of alginate and biosurfactants that maintain the architecture of the biofilm by contributing to the formation of internal cavities in the mature biofilm. It allows to have a good flow of water and nutrients from the medium, which leads to a restoring process of the hydroelectric medium balance for reducing the effect of the osmotic shock on the biofilm and the cells.[37] The overproduction of alginate and rhamnolipids is responsible for the formation of the mucoid population at the end of the chronic stages in *P. aeruginosa*.[37] Alginate and rhamnolipids detected by the agar diffusion method (Figure 4) are the main

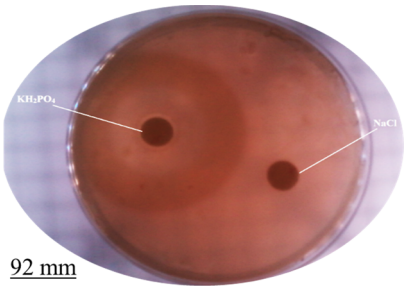


Figure 4. Results of 12 h of KH_2PO_4 and NaCl bacteriostatic and bactericidal effect.

barriers resistance protectors for *P. aeruginosa*, particularly during the invasive infectious process in the host cells. The phenomenon of the hydroelectric balance results from the presence of a large amount of hydronium ions from KH_2PO_4, which acidify the growth medium and modify the electrical charge of the bacterial membranes, creating a disruption at the levels of the interactions such as cell−cell, cell−surface, and biofilm−surface. This disruption of the interactions allows to maintain the compactness and the stability of the biofilm because of the dispersion of the colonies and the destruction of the biofilm by cellular degeneration.

From Figure 2, it was noticed that the biofilm increased homogeneously along the liquid surface. In the presence of KH_2PO_4, the maximum of the biofilm was formed at the liquid−air interface. This effect can be related to the mobility of saline ions, favored by a large amount of water and a high electrical conductivity of the medium. The growth of the biofilm is strongly limited by the competitiveness of the saline ions for the molecules of water against the strain; the access to the carbon substrate and the essential nutrients is limited, which causes a blockage of the secondary metabolism of the protective agent secretion and a stabilization of the biofilm from an average concentration of MBEC $\geq$ 3.6%.

Indeed, the difference in the process of biofilm eradication with KH_2PO_4 resided in the acidification forces that the ionic dissociation caused (liquid medium). The pH of this medium showed a high acidity against NaCl, thus promoting the eradication of the biofilm and the decline of the cell growth with a concentration of MBEC = 3.6%, RIB = 91.12%, and BKQ = 50.90%. The action of KH_2PO_4 on the biofilm resulted from the synergy between the stimulating process, the hypertonic saline force, the competitiveness for the water molecules, and the acidification of the medium by the ionic dissociation.

The synergistic action of KH_2PO_4 in the biofilm eradication corroborated well the correlation of the results obtained in the liquid selective medium, where BKQ = 60.51% and RIB = 98.51%. Also, the synergistic action strength of KH_2PO_4 caused the inhibition of quorum sensing by preventing the regulation of the synthesis of the virulence factors and the structured formation of the biofilm. In contrast to the solid environment, the structure of the biofilm was favored by the presence of physical barriers because of various secretions of xenobiotic polysaccharides.[37,42] Moreover, this synergy was at

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the origin of the absence of any cellular accumulation from the concentration of MBEC ≥3.6% during the experiments and favored the preservation of the hydroelectric balance of the environment. This is an important therapeutic element because it leads to the clearance increase of the mucus and decreases the exacerbation of the bronchitis or the cystic fibrosis.

In their study, Clunes and Boucher[41] and Michon et al.[17] observed a similar behavior in *P. aeruginosa* facing a hyperosmotic shock because of the hypertonia of the medium. However, no secretion was observed in the case of NaCl in the solid medium except a depigmentation that is characteristic of the colonies by inhibition of the secretion of pyocyanique and pyoverdine. The absence of secretion of the pyocyanine reduces the viscosity of the biofilm, which influences the physicochemical interactions of the biofilm matrix with the environment and makes the cellular aggregations difficult.[37,43] This reduction of the rate of humidity of the medium by dehydration conditions causes the degeneration and the destruction of the biofilm over the time because the access to water molecules is limited by the chlorine action.

From Figure 2, the results obtained on the selective liquid environment containing NaCl showed a zone of low resistance of the strain *P. aeruginosa* in the range of concentration of 0.3−1.2%. In this range, the maximum formation of the biofilm was located on the surface of the liquid and on the liquid−air interface. The mechanism of action of NaCl was carried out by the ionic competitiveness of the chlorine for water molecules against the strain that acidifies the medium and by the accumulation of cytoplasmic Na^+, which inhibits the biofilm growth. For high concentrations of NaCl, above 0.3%, the formation eradication and the biofilm propagation were proportional to the increase of the hypertonicity of the medium with an average MBEC ≥3.6%, which allowed to block the bacterial duplication in the individual state as well as in the biofilm state. The biofilm was characterized by a strong resistance to the antimicrobial effect of salts. From MBEC = 4.2%, the resistance was limited and the eradication process was optimal. There was an accumulation of cytoplasmic sodium that provokes the disorder of the Na^+ membrane pumps, accompanied by a leakage of the hyaloplasmic flow toward the extracellular space, leading to the desiccation of the biofilm.

The leakage of the hyaloplasmic flow is identical for the two environments tested with NaCl. It is characterized by the depigmentation of the biofilm, which leads to a hyperosmotic shock and has for consequence the destabilization of the periplasmic pressure and induces cellular lysis individually, as well as the eradication of the biofilm during the incubation time.

In the liquid environment, the hyperosmotic shock, as well as the biofilm physiology provoked, is common for NaCl and KH_2PO_4. In addition, sodium and potassium cytoplasmic accumulation characterizes a turgescence by the intracellular influx, leading to a disruption between the cytoplasm and the membrane plasma by the denaturation of structural proteins, resulting in cellular lysis by degeneration. Moreover, even for bacteria that could adapt to a hyperosmotic shock, sodium and/or potassium intracellular accumulation established strongly a denaturing element for proteins, plasmids, and bacterial enzymes by blocking all the cellular metabolisms and the life cycle.

The process of acidification by the chlorine and hydronium ions affects considerably the mobility and the adhesion of the strain by causing a dysfunction of the pili, flagellum, fimbriae, and also the secretion of toxins associated with fimbriae in the invasive infectious process. Similar observations were made in *P. aeruginosa* in hyperosmotic shock caused by the hypertonic saline solutions.[13,17] Figure 1B,C demonstrates that MICs for 90% of the biofilm were achieved at MBIC = 3.9 and 4.2% with RIB = 90.55 and 90.80% for KH_2PO_4 and NaCl, respectively. Contrarily, owing to the inhibition, the biofilm eradication process by NaCl showed a lower concentration in comparison with that by KH_2PO_4. From Figure 1, it was noticed that MBEC = 3.3% with BKQ = 60.24% and MBEC = 2.7% with BKQ = 55% for KH_2PO_4 and NaCl, respectively. This difference is due essentially to the process of the stimulation of the biofilm growth by KH_2PO_4 at each concentration. It allows *P. aeruginosa* to produce various xenobiotic compounds in the environment. The isolation of these compounds with 10% of salts allowed to demonstrate that they possess the capacity to lower the surface biosurfactant tension using the method of Young.

Tahzibi et al.,[44] Christova et al.,[45] Gunther et al.,[46] and Emerson et al.[47] showed that within the framework of the screening test and the identification of the production of biosurfactants by *P. aeruginosa* in a solid medium, KH_2PO_4 stimulates the synthesis and the secretion of biosurfactants (KH_2PO_4 = 13.34%) by diffusion. Indeed, the test of confirmation of the biosurfactant secretion by the biofilm in the presence of KH_2PO_4 showed the formation of three zones: the first describes a bactericidal effect, D = 1.2 cm.[48] The second zone characterizes the inhibitory effect, D = 3.5 cm, and the third, of a dark-blue coloration, characterizes the stimulating effect on the biofilm growth by the secretion of biosurfactants, D = 4.2 cm (Figure 4). The dark-blue coloration indicates the presence of rhamnolipids beyond the inhibition diameter. Rhamnolipids play a crucial role in the development and the biofilm preservation. Biosurfactants produced by the biofilm favor the adhesion on the surface of the solid and participate in the mobilization and solubilization of nutrients in order to ensure the continuity of all metabolic reactions that maintain the biofilm stable by protecting it from the hypertonic stress, thus facilitating the virulence during the infection.[34] With regard to the results presented in Figures 1 and 2 on the biofilm inhibition and eradication by hypertonic saline solutions, it was demonstrated that the efficiency and the performance of the tested salts according to their main action process on the biofilm are independent of the medium type.

3. CONCLUSIONS

The comparative study of two salts, KH_2PO_4 and and NaCl, used for the eradication of a biofilm of *P. aeruginosa* in a solid and liquid environment in vitro, allowed to monitor the kinetic of the inhibition and of the eradication of this pathogenic agent responsible for several nosocomial infections in a hospital environment. The obtained results on the eradication of the biofilm showed an antimicrobial property of KH_2PO_4 for a short duration of exposure time of 48 h with very high rates of inhibition and/or eradication in comparison with NaCl.

Furthermore, different mechanisms of actions in the process of the biofilm eradication were proposed for the two salts. Regarding KH_2PO_4, it showed a strong inhibition before the eradication "defrosting", whereas NaCl inhibited moderately before the eradication by "desiccation".

Contrary to NaCl, synthesis of biosurfactants and their secretion were observed in the case of KH_2PO_4. In addition,

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the agar diffusion and successive dilution tests allowed to correlate the identification of the mechanisms of action in both tested environments and also the primordial role of the biosurfactants and their stimulus. The statistical, variance (analysis of variance, ANOVA) and *K*-nearest neighbor, analysis of the results obtained showed the preponderance of the correlation between the concentrations of the tested hypertonic saline solutions, the inhibition rate, and/or the rate of eradication of the biofilm.

4. MATERIALS AND METHODS

4.1. Antimicrobial Effect Monitoring. The strain used in this work was isolated from the hospital-contaminated ground of Medea-Algeria. The "cetrimide agar" (European Pharmacopeia 4.7) was the selective medium for the *P. aeruginosa* phenotypical and physical identification used in this study. For this purpose, two *P. aeruginosa* strains, ATCC 27853 and ATCC 9027, were used in the same conditions. All the elements were successively added and then heated until boiling under a constant agitation for the homogenization to assure a complete dissolution before the sterilization in the hanging autoclave for 15 min at 121 °C.[29] The solid culture medium was used for the study of *P. aeruginosa* biofilm behavior in a solid−air interface in a static microplate condition. On the one hand, the antimicrobial salts were added to the selective medium and KH_2PO_4 in Petri dishes for 48 h of culture. On the other hand, NaCl at the same concentration and during the same incubation time was used in order to compare the effects of both salts.[17−22] The monitoring of the inhibition and/or the killing of the biofilm was done by a digital photogrammetric method in a stove for 48 h of incubation at 37 °C.[30,31] A digital photogrammetric method was applied according to the principle of a correlated imaging method by using a smartphone for the *P. aeruginosa* biofilm behavior in an interface solid−air optical surface density.

The liquid medium was a cetrimide medium because it allows to guarantee the preservation of the selectivity to the followed growth without disturbance because of a biological contamination. All elements were added and then completed with KH_2PO_4 or NaCl to monitor the biofilm behavior over a period of 48 h at 37 °C with the same concentrations as the solid medium. At the end of 48 h, 4 mL was taken after the homogenization of the medium. The biofilm growth was measured by the spectrophotometric method at 600 nm.[32] To estimate the effect of NaCl and KH_2PO_4 on the *P. aeruginosa* biofilm, 52 Petri dishes were prepared containing the medium "cetrimide agar" and the antibacterial salts in hypertonic concentrations in the range of 0−7.5% with a step of 0.3%.[17] The sowing of the solid medium was conducted by the technical sowing deposit colonies on the surface of the agar. It consists in taking by means of a pastor pipette a colony stemming from a young culture and then putting it down on the agar surface by ensuring that all biomass is fixed.

It also allows to have a massive bacterial density on the agar surface and thus favors the biofilm development in the optimal conditions of growth. Therefore, the mobility of the strain allowed it to colonize all agar surfaces.[17] After sowing, the preparations were incubated in a stove for 48 h at 37 °C. The estimation of the propagation of the biofilm of *P. aeruginosa* was determined by applying the digital photogrammetric method, a technique that allows to extract from images quantitative and qualitative information of an object. According to Kellett et al.,[16] the growth rate was estimated according to the propagation of the biofilm density calculated from the Petri disks by the surface occupied between the Petri dishes of 0% hypertonic salts and all Petri dishes of hypertonic salts after 48 h of culture. Therefore, the method used to calculate the surface is based on the application of a multimedia software (NIJ ImageJ. 16.0) in order to obtain surfaces in pixels. The number of pixels corresponds to the density of the surface of each biofilm. The density of the eradication of the biofilm on Petri dishes was calculated by means of a smartphone of type Condor C8 device of dimension (7.7 × 15.4 × 1.0 mm) with a screen resolution of 1280 × 720 pixels.[24] All collected data were treated by SPSS.20, Excel 2016, Origin Pro. 8, and NIJ ImageJ. 16.0 softwares.[30,31]

The monitoring of the kinetic in the liquid medium was done by measuring the optical density between the initial and the final time of 48 h at 600 nm.[33] The sowing of all tubes was performed with an inoculum volume of 0.1 mL (1% v/v) of a new obtained bacterial suspension after 18 h of culture in a selective medium "cetrimide agar" and incubated in a horizontal position to increase the contact area with the air oxygen at 37 °C. At the end of the culture, 4 mL samples were taken in each tube to determine the viable biomass against concentration. First, the medium culture was well shaken to homogenize bacterial concentrations before every experiment. The number of tubes and the antibacterial hypertonic concentrations were identical to those of the solid medium culture.[11] The agar diffusion method was used to confirm the role of rhamnolipids produced by *P. aeruginosa* in the formation and the preservation of the biofilm by means of disks impregnated with hypertonic solutions of concentration of 10% after 15 min and deposited on a "cetrimide agar" medium previously inoculated with a bacterial suspension and tested using the dark-blue method in the screening of the biosurfactants.[8,34,35]

4.2. Determination of MIC and MBC. The technique of successive dilutions in colony forming unit (cfu) was used to determine the MIC and the MBC.[11,36,37] The optical density from the nourishing broth of 10 mL was measured and normalized in 6×10^8 bacteria/mL equiv in OD unit at 600 nm during an incubation time of 24 h at 37 °C. A series of 74 test tubes containing a sterile broth of 10 mL were incubated. The concentration of KH_2PO_4 was in the range of 0−7.5% with a step of 0.3%. The same method was used for NaCl. The initial inoculum was about 300 cfu diluted in 1/10th for every test from a new culture from the feeding broth of 18 h at 37 °C.[35] The determination of the MBC was performed by using the resulting test tubes of MIC assays and an inhibition supplied by KH_2PO_4 or NaCl.[11] The inoculums of test tubes were seeded on a solid medium culture to obtain a bactericidal effect of KH_2PO_4 or NaCl for 24 h incubation period at 37 °C. The initial inoculum of 300 cfu was diluted to 1/10th for each test tube for a new culture from broth feeding for 18 h at 37 °C.[35] The MBC was determined using the resulting test tubes of MIC assays that have an inhibition supplied by KH_2PO_4 and NaCl.[11] The inoculums were inoculated on a solid medium culture to obtain a bactericidal effect of KH_2PO_4 and NaCl for 24 h of incubation at 37 °C.

4.3. Data Treatment. This study used the macroscopic focal photogrammetry method described by Broderick et al.,[30] Saimmai et al.,[31] and Slama[32] to estimate the density of the biofilm as a function of the focal length between 0.01 and 0.1 m. The pictures of all the Petri dishes were taken by

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considering the same calibration parameters. They were taken with high accuracy and different backgrounds, allowing to facilitate the distinction between the colonies and their environment.[23,26−32] The photogrammetric method cannot reconstruct visible surfaces because the method uses an approach of an uncalibrated camera. Dimensional references are necessary to convert the reconstruction into real dimensions. The quality of the reconstruction depends on the resolution of the image, the target, and the number of images.[30] To obtain the same surface in pixels for all Petri dishes treated, each taken image was instinctively returned to the size of Petri dishes in order to eliminate the periphery following the same ratio height/width. This step is very important to guarantee the accuracy of the measurements related to the biofilm density. The sizing was done by the tool "size of image" of Photoshop. It allows to compare better the different Petri dishes and to translate the images into raw algebraic values of the occupied surface in percentage.[38]

4.4. Statistical Analysis. All experiments were replicated three times on different days. According to Chhibber et al.,[39] the estimation of the biofilm density was the surface density and the transformed optical density. The effect of different treatments on biofilm eradication was assessed by the test of variance ANOVA and $P < 0.05$ was considered significant.[39] The data were analyzed using SPSS.20, Origin Pro.8, and Excel 2016. For the reproducibility of the obtained results, all the experimental assays were carried out three times and all duplication was carried out in double samples in the same conditions. The objective of this replication was to constitute a statistical cohort in order to carry the aberrant data using SPSS.20−Data Mining (*K*-nearest neighbor analysis and predictive correlated modeling modules). This statistical method showed a guarantee of the reproducibility of the obtained results. Thus, on all three corrected assays and when consigned by the *K*-nearest neighbor analysis, an ANOVA statistical analysis was carried out using Origin Pro 8 software. All data were modeled by the predictive correlation module of SPSS-Data-Miner. The statistical analysis parameters such as sum of square, mean square, distribution function *F* value, error of distribution function Prob > *F*, correlation data R^2, and Adj. R^2 in confidence interval CI ≥ 95% were studied according to Bassirou et al.[40]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02733.

Comparatives results of NaCl and KH_2PO_4 according to the concentration of the cetrimide agar medium; statistical analysis and ANOVA of all biofilm densities from the solid and liquid medium; results of the *K*-nearest neighbor analysis (CPS: case processing summary); and *K*-nearest neighbor analysis and predictive correlated modeling (characteristic "central", type "learning and retention" and $K = 3$) applied from KH_2PO_4 and NaCl in the solid and liquid environment, respectively (PDF)

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Notes

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■ ABBREVIATIONS

ANOVA, analysis of variance; BKQ, biofilm killing quotient; CFTR, cystic fibrosis transmembrane conductance regulator; CPS, case processing summary; HTS, hypertonic salt; CI, confidence interval; KH_2PO_4, potassium bihydrogen-phosphate; MIC, minimum inhibitory concentration; MCB, minimum bactericidal concentration; MBEC, minimum biofilm eradication concentration; MBIC, minimum biofilm inhibitory concentration; *N*, number of observation; NaCl, sodium chloride; OD, optical density; RIB, biofilm inhibitory rate

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