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Effects of Environmental Conditions on Growth and Permanence of *Pseudomonas aeruginosa* in Bottled Water

Gholam Reza JAHED KHANIKI* Ebrahim MOLAEE AGHAEE Mahmoud ALIMOHAMMADI Mohammad Hadi DEHGHANI
Department of Environmental Health Engineering, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

*Corresponding author:
E-mail: ghjahed@sina.tums.ac.ir

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Abstract
Pseudomonas aeruginosa is considered as a process quality management indicator. Its occurrence reveals an environmental pollution at either the water source or at bottling process area. The present paper aims to study the effects of environmental conditions on the growth and survival of this bacterium in bottled water. Initial bacterial load in samples before inoculation and subsequently at various intervals was measured by membrane filtration technique. Microbial suspensions containing *Pseudomonas aeruginosa* were prepared to inoculate at least 10^2 cfu/ml microorganisms in each bottle. Samples were stored at 4ºC, 20 ºC and 37 ºC, as well as outdoors in darkness and in sunlight for 0 (immediately after inoculation), 3, 7, 14, 28 and 50 days. Results demonstrated that bacterial population increased by the 3rd day in all of the conditions, and that samples stored at 37 ºC showed the highest increase. The most substantial reduction in the amount of bacteria was observed at 4 ºC and for the samples in sunlight, so that after 50 days it was as low as 0.1 of the initial amount. Room temperature seemed to have no noticeable effect after 50 days. It was shown that high temperatures could lead to an increase of *Pseudomonas aeruginosa*, while low temperatures and sunlight in cold weather could decrease its concentration.
Key words: *Pseudomonas aeruginosa*, Bottled water, Storage, Environment, Light, Temperature.

INTRODUCTION

Consumption of contaminated water causes illness in human. According to the World Health Organization (WHO), over 1.8 million people, who are mostly children, die annually due to water-borne diseases, which have been one of the leading and still prevalent mortality causes [1, 2]. Regarding the increase of bottled water consumption instead of municipal tap water, its microbial safety must be more controlled [3]. The rationales behind higher consumption of bottled water, on the one hand, are consumer's concern over environmental water pollution, municipal water problems such as off odor and taste, high amount of fluoride and chloride and, on the other hand, suitability of bottled water for those with immunity system defects and general conception of bottled water as a safer product [4-6]. Quick bacterial amplification after bottling could be due to oxygenation of water throughout processing, increase in bottles' surface area, rises in temperature owing to inappropriate storage and nutritional compounds of bottles. Furthermore, undesirable storage conditions, could lead to unfavorable changes in color, odor and taste, and in more severe cases microbial load will result in microbial outbreaks [1, 7, 8].

P. aeruginosa, as a prevalent bacterium in the environment, can cause renal, soft tissue, respiratory system and some other systematic infections, particularly in patients with immunity system defects, and could also lead to cystic fibrosis and gastro enteric outbreaks [9-10]. Ullah et al. (2012) studied antimicrobial resistant *P. aeruginosa* in contaminated fresh spring water and announced that bottled water can be contaminated with this bacterium; therefore, it could be a potential health risk for humans. Its absence suggests the uppermost negative percentage of other indicators in tap water (89.7%) and bottled water (56.2%) [11]. Furthermore, there is a positive relationship between the presence of *P. aeruginosa*, fecal coliforms and staphylococcus species [12]. *P. aeruginosa* is a predominant environmental organism, since it is an oligotrophic and opportunistic pathogen and is also an indicator of process management in production of bottled water plant. Its presence shows that water source has been polluted by organic compounds and/or the pollution has arisen throughout bottling process [5, 13]. Additionally, *P. aeruginosa* has a synergistic effect on pathogenic microorganisms such as salmonella and can contribute to their survival in distilled water for 140 days [14]. Moreover, *P. aeruginosa* is highly prone to growth in bottled water, does not need any vitamin or amino acid and can survive with various carbon sources [15]. According to Lecrec and Moreau (2002) bacteria have a viable but non-cultivable state in bottled water. After bottling, the amount of viable microorganisms increases quickly and reaches 10^4-10^5 cfu/ml in 3-7 days [16]. Implementation of hazard analytical critical control points (HACCP) system in stores, demonstrated that storage for one month does not have any negative effect on microbial safety of bottled water because of good transportation and storage conditions [6]. There are two principal concerns about *P. aeruginosa*: it is an indicator of inadequate environmental monitoring in the bottling plant and also an opportunistic microorganism with minimal nutritional demands [16]. The present paper aims to study the effects of environmental conditions on permanence and growth of *P. aeruginosa* in bottled water.

MATERIALS AND METHODS

Source of materials and sample preparation

20 Bottled waters from different brands with high consumption rates were purchased from Tehran market, Iran. In addition, it was considered to select the brands which were produced in different environments and regions of the country. In order to determine the concentration of bacteria in the collected samples before the inoculation, 250 ml of each sample was filtered by standard membrane filtration technique, and was cultured in the following media. All materials were provided by Merck Company, Germany.

Chemical tests for bottled water samples

Chemical examinations were performed by Ion Chromatography (IC) for anions and cations and also other procedures according to standard methods for the examination of water and wastewater, USA; 21st edition.

Inoculation of *P. aeruginosa* into bottled water and microbial examination

Standard strain of *P. aeruginosa* ATCC 9027 in Trypticas Soy Agar (TSA) medium was surface-cultured and incubated at 37 ºC for 48 hrs. Next, by using McFarland standard solutions, the microbial suspensions were prepared in physiologic serum with intended concentrations for inoculation to bottled waters. Defined amount of suspension containing *P. aeruginosa* from the prepared serial dilutions was inoculated to bottled water samples and, to assure the inoculated amount of at least 10^2 microorganisms in each sample, they were immediately cultured on Cetrimide agar (C.A) by standard membrane filtration (0.45 µm) that was connected to a vacuum pump, and after culturing on Cetrimide agar and incubation at 37 ºC for 48 hrs, colonies were enumerated. In order to verify that whether the bottled water condition has prevented the growth of some microorganisms in Cetrimide agar, a resuscitation step including pre-incubation of membranes in TSA was conducted for 1 hr at 37ºC and then inoculation on Cetrimide agar was employed [13]. Confirming tests, including gram coloring, catalase and oxidase tests, were conducted for unidentified and suspicious colonies.

Time and storage conditions of bottled water samples

Different storage conditions for the samples were as follows: refrigerator at 4ºC, room temperature at 20ºC, incubation at 37ºC, and outdoor condition (average 14.5ºC) both in darkness and sunlight at different intervals of 0 (instantly following inoculation), 3, 7, 14, 28 and 50 days. Blank samples, i.e., bottled waters without inoculation in the described conditions were used and all tests were carried out with two series of samples and also in duplicates.

Statistical analysis for data

Data were entered into the software and statistical analyses including Block analysis and non-parametric Wilcoxon and Friedman tests were performed using SPSS (version 16.0). A probability level of $p < 0.05$ was considered statistically significant.

RESULTS

Chemical characteristics

The chemical properties of all different bottled water samples were measured in order to have better consideration of the examined samples. Chemical properties of examined bottled water samples are presented in Table 1. The mean and standard deviation of pH, NTU, EC and TDS in examined bottled water samples were 7.48±0.07, 0.23±0.007, 370.4±4.52 µs/cm and 204.43±3.85mg/l, respectively. Also, the content of fluoride(F), chloride(Cl), nitrite(NO_2), nitrate(NO_3), sulfate(SO_4), sodium(Na), potassium(K), calcium(Ca) and magnesium(Mg) in examined samples were 0.14±0.03 mg/l, 3.30±0.02 mg/l, 0.012±0.004 mg/l, 7.36±0.23 mg/l, 17.41±0.13 mg/l, 5.70±0.09 mg/l, 0.73±0.06 mg/l, 44.12±16.08 mg/l and 9.95±0.34 mg/l, respectively.

Table 1. Chemical properties of bottled water samples

Mean and Standard deviations of chemical parameters						
Samples no.	F mg/l M±SD	Cl mg/l M±SD	No_2 mg/l M±SD	No_3 mg/l M±SD	So_4 mg/l M±SD	Na mg/l M±SD
20	0.14±0.03	3.30±0.02	0.012±0.004	7.36±0.23	17.41±0.13	5.70±0.09

Mean and Standard deviations of chemical parameters							
Samples no.	K mg/l M±SD	Ca mg/l M±SD	Mg mg/l M±SD	pH M±SD	[a]NTU M±SD	[b]EC µs/cm M±SD	[c]TDS mg/l M±SD
20	0.73±0.06	44.12±16.08	9.95±0.34	7.48±0.07	0.23±0.007	370.4±4.52	204.43±3.85

[a]NTU: Nephelometric Turbidity Units
[b]EC: Electrical Conductivity
[c]TDS:Total Dissolved Solids

Effect of different conditions on growth and permanence *P. aeruginosa*

Prior to the inoculation, all the samples were analyzed, through microbial culturing as well as filtration and confirming tests, to make sure they initially contained no *P. aeruginosa*. Since there was not any significant difference in the growth and permanence of *P. aeruginosa* in all the samples after inoculation as indicated in Figures 1 and 2. In the 3rd day the amount of microorganisms showed an increase in all the samples. The highest growth was observed at 37 ºC. In the 7th day, bacterial population showed a decrease in all the samples except for those stored at 37 ºC, and also the most significant decline belonged to samples at 4 ºC and those stored outside and exposed to sunlight. However, the bacterial concentration of samples still increased at 37 ºC by the 7th day. Reduction rate remained almost steady until the 14th day in all the samples, whereas the samples stored at 4 ºC, 20 ºC and darkness did not show any noticeable reduction and had an almost consistent rate. Other samples especially those at 37 ºC had a decreasing rate by the 28th day. From the 28th to 50th day all the samples except those at 20 ºC maintained a decreasing trend of bacterial growth, for which the samples stored at 37 ºC and outside both in darkness and in sunlight showed a sharper rate. The samples stored in room temperature stayed in an almost stationary state from the 7th to 50th day, and at the end of the study the bacterial population difference was not significant in comparison with the initial inoculated amount ($p < 0.05$). After 50 days, bacterial concentration in samples stored at 37 ºC was already twice its value in day 0, but in samples stored at 4 ºC and outside (both sunlight and darkness), it was much lower than the initial inoculation amount, 0.1 of the amount for the samples stored at 4 ºC and sunlight. Figure 1 shows that the difference was not significant in the initial inoculated concentrations for all the samples ($p > 0.05$). Nevertheless, the difference of bacterial concentrations for the other days in different temperatures was significant ($p < 0.05$), for which the lowest concentration was observed at 4 ºC, with the highest concentration being detected at 37 ºC.

Also, the difference of bacterial content in various days was significant at 4 ºC ($p < 0.05$) and the bacterial concentration was 0.1 of the initial quantity. At the temperature of 20 ºC the bacterial content was already constant after the 7th day until the last day of study and still was higher than the initial inoculation amount. At 37 ºC the difference of bacterial population was significant ($p < 0.05$), for which the highest amounts were observed in days 7, 3, 14, 28, 50 and 0 respectively.

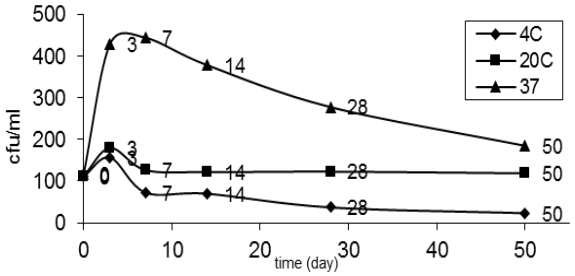

Figure1. Changes in growth and survival of *Pseudomonas aeruginosa* in different temperature and time conditions

Figure 2 shows the effect of sunlight and darkness on the growth and survival of *P. aeruginosa* in outside area. Results attested to the difference of bacterial content in the 3rd day ($p < 0.05$), for which the amount of *P. aeruginosa* was higher in the sunlight than in the darkness. At other intervals the differences was significant too ($p< 0.05$); however, the bacterial concentration was higher in the darkness condition than in the sunlight. Moreover, throughout the storage time in sunlight, bacterial growth was more prohibited.

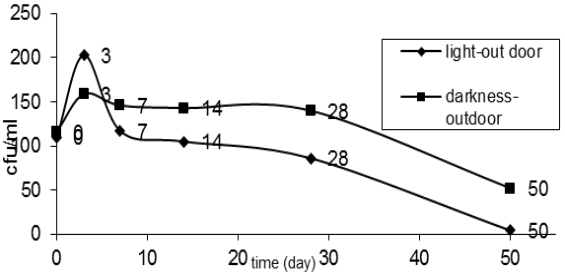

Figure 2. Effects of sunlight and darkness on growth and survival of *Pseudomonas aeruginosa* in outside

DISCUSSION

Bottled water can be contaminated to P. aeruginosa. This microorganism belongs to a large group of free-living bacteria that there are in water and the environment. This bacterium is important as aspects of public health concerns. It can enter to human body through drinking water and induce some infections, especially in immunosuppressive people. It can cause different diseases such as endocarditis, osteomyelitis, pneumonia, urinary tract infections, gastrointestinal infections, meningitis, and septicemia. This bacterium can occur in drinking water and bottled water. It needs the least nutrients and can survive in water [17]. Usually, bottled water store in shops for many times after production until selling and if it has been contaminated to P. aeruginosa, this bacterium grows at environmental conditions. Time and storage condition is important on growth and permanence of P. aeruginosa in bottled water. Legnani *et al.*, (1999) reported that in the first 2 days of their study, bottled waters with lower total dissolved solids (TDS) and stored in room temperature demonstrated a more rapid growth, followed by a decrease. Moreover, in samples with higher amounts of saline, the growth phase was almost longer and remained for 3 days. Generally, the concentration of bacteria increased until the 4th-5th days and then reached a stationary phase, followed by a gradual decline [13]. In present study, we did not observe any significant difference in the growth curve in samples with different TDS and saline content; however, the other results were already the same as the findings mentioned above. In another research, bacteriological permanence and growth kinetic of *P. aeruginosa* was investigated in bottled water, and *P. aeruginosa* was found in 12% of the bottles. Duplicating time of the microorganism in the presence of aerobic flora was 26 hours in room temperature [18]. In the present study, the highest amount of bacterial population was observed after 3 days in all the conditions, and the duplication was just seen at 37 ºC after about 72 hours. Nsanze et al. (1999) mentioned that storage of bottled water at 4 ºC decreased microbial propagation, while at 25 ºC and 37 ºC most of the microorganisms multiplied, and at 42 ºC started to decrease [14]. In this paper, results for the samples stored at 4 ºC and 37 ºC were the same; however, at room temperature with the average of 20 ºC the bacterial population was almost constant after the 3rd day. Raj (2005)

announced that the total bacterial count of bottled water, which was monitored using standard microbiological techniques over 48 hours at 37ºC, had increased, but markedly reduced at cooler temperatures [19]. In other studies, Pseudomonas strains constituted the largest population of bacteria in still bottled mineral water after 3 weeks of storage in the ambient temperature and were mostly resistant to antimicrobial factors. Also, the largest amount of microbial strains in bottled water belonged to *P. aeruginosa* [4, 5]. Mahmud et al. (2009) investigated *P. aeruginosa* in 238 bottled mineral water samples, produced by different manufacturing companies and purchased from the local market in Bangladesh. They detected that 59 (25%) of the 238 bottled mineral water samples contained *P. aeruginosa* [20]. Baumgartner and Grand (2006) reported that *P. aeruginosa* had been identified in 25% of the examined samples [21]. However, in the present study, none of the purchased bottled waters contained *P. aeruginosa* and the total count was lower than the standard limits. As mentioned before, the brands were among the most popular ones; therefore it is probable to count *P. aeruginosa* in other bottled water brands in the market.

In general, low temperatures reduce the growth of *P. aeruginosa*. Regardless of the period of storage, bacterial growth and concentration increased in higher temperatures. The outdoor storage condition, with the measured ultraviolet (UV) irradiation of about $0.003 w/m^2$ in comparison with darkness condition, led to a decrease in bacterial population and the same results for storage at 4ºC was observed by the end of the study. Also, some chemical materials such as phthalates compounds are present in almost all plastic equipment [22] and packaging the bottle of water [23]. Sunlight exposure of bottled water could result in the migration of terephthalate compounds from the polyethylene terephthalate (PET) bottles into the water and this poisonous chemical materials in water may led to decrease microorganisms and it can be a reason for decreasing the bacteria.

CONCLUSION

In general, low temperatures reduce the growth of *P. aeruginosa*. Regardless of the period of storage, bacterial growth and concentration increased in higher temperatures. The outdoor storage condition can led to led to a decrease in bacterial population in comparison with darkness condition, but the hazardous materials may release from bottle wall into water. Thus, it is strongly recommended not to store bottled water in sunlight and hot weather for a long time. Other inappropriate storage conditions such as warm and enclosed areas like inside cars, parking lots, bags, etc., which provides growth conditions for several bacteria including *P. aeruginosa* could be further studied.

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