Bactericidal activity of chlorine on some pathogenic bacterial strains isolated from Nile water

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Abstract

The principal factors that influence disinfection efficiency are disinfectant concentration, contact time, temperature and pH. The main objective of this study was to use chlorine gas for inactivation of four bacterial strains isolated from inlets of some drinking water treatment plants in Sharkyia governorate. The bacterial isolates were identified up to species level after being submitted to morpho- physiological as well as biochemical tests and confirmed by using BIOLOG GN III. The results revealed that the removal percentages of *Escherichia coli* O157:H7, *Staphylococcus epidermis, Pseudomonas aeruginosa* and *Bacillus subtilis were* 99.98, 100, 99.92 and 98.83% respectively, after 10 min contact time. The breakpoints of *E. coli O157:H7, Staphylococcus epidermis, Pseudomonas aeruginosa* and *Bacillus subtilis* were observed at chlorine doses 1, 2.2, 1.4 and 0.6 mg/L, respectively. Consequently, *Staphylococcus epidermis* and *E. coli* O157:H7 showed more resistant to chlorination than *Pseudomonas aeruginosa* and *Bacillus subtilis.*

Key words: disinfection, bacteria, chlorination, breakpoint, Nile water.

Introduction

Drinking water disinfection commonly involves the use of chlorine in the treatment process, i.e., for pre-treatment (to induce a primary disinfection at the beginning of the treatment process) and/or for post-treatment (to maintain a disinfectant residual in the distribution system) **(WHO, 2011)**. Therefore, it is important to study the efficiency of chlorine on some selected bacteria. Owing to its low cost; chlorine is globally the most used chemical oxidant for drinking water disinfection. Despite its low activity on microorganisms in biofilms, chlorine can lead to a significant removal of the majority of planktonic bacteria **(Le Chevallier *et al*., 1988; Bois *et al.*, 1997).** Thus**,** chlorine plays an important role in limiting the growth of heterotrophic microorganisms when added near the end of the treatment process, i.e., before water release in the distribution system **(APHA, 2011)**. As a chemical oxidant, though less reactive than ozone, chlorine can transform numerous inorganic and organic micro- pollutants found in water (e.g. Fe

(II), As (III), $NO2^-$, phenols, pesticides, pharmaceuticals, etc.) **(Lahoutifard *et al.*, 2003; Diurk and Colette, 2006; Dodd *et al.*, 2006).** Chlorination usually represents an efficient process to remove/transform inorganic micro-pollutants. However, due to the potentially harmful chlorinated transformation products; chlorination is usually not applied for oxidation of organic micro-pollutants (**Richardson, 2005)**. Similar to other disinfection processes, chlorination presents certain disadvantages in spite of its broad use and its benefits for the improvement of microbial water quality due to its pH-dependent aqueous chemistry, various species of chlorine (HOCl, ClO^-, Cl2, etc.) may be present in solution **(Dore´, 1989).** These forms of chlorine show significant differences in their reactivity with microorganisms and micro-pollutants. Therefore, variability in oxidation or disinfection efficiency can be observed depending on the pH of the water. Chlorine interacts with dissolved natural organic matter.

Chlorine residuals in drinking water have long been recognized as an excellent indicator for studying water quality in the distribution network (**Lienyao *et al*,. 2004**). In the absence of a disinfectant residual, microorganisms in the distribution network will be recovered at high levels. The presence of any disinfectant residual reduces the microorganism level and frequency of occurrence at the consumer's tap **(Olivieri *et al.*, 1984).** U.S standard agency specifies a minimum level of 2 mg/L total chlorine residue be required to prevent biofilm growth and protect water quality deterioration. Keeping residual chlorine at a certain level in tap water is effective not only in improving sanitary conditions but also in suppressing the regrowth of microorganisms and preventing the formation of biofilms on the internal surface of distribution pipelines **(Kitazawa, 2006).**

Chlorine effectively inactivates the majority of organisms that cause diseases in humans like at 0.2 mg/L chlorine concentration for 3 minutes cause 99.99% reduction of *Escherichia coli* and at 0.5 mg/L chlorine for 6 minutes reduces 99% *Salmonella typhi* **(Ram and Malley 1984; Fass *et al*., 1996).** Addition of chlorine in different water treatment plants is a common practice, but it is not sufficient to ensure the safety of water. Regular testing is essential to ensure that adequate free residual chlorine is still present in the treated water. The maintenance of chlorine residue is needed at all points in distribution system supplied with chlorine as a disinfectant (**Munavalli and Mohan Kumar, 2003).**

So**,** the present work aimed to study the bactericidal activity of chlorine on some bacterial strains with regards to the dosage of disinfection and contact time.

Materials and Methods

All the chemicals used in the present work including starch, potassium dichromate ($K_2Cr_2O_7$) **(**0.025 N), sodium thiosulfate ($Na_2S_2O_3$.5H2O) (0.025 N), phosphate buffer solution, N, N-diethyl-p-phenylenediamine (DPD) indicator solution were of analytical purity grade and prepared as mentioned in **APHA (2005).**

Water sample and sampling sites:

Water samples were collected from drinking water treatment plants (DWTPs) receiving Nile water in Sharkyia governorate. Bacterial strains used in the present work were isolated from inlets of these drinking water treatment plants.

Isolation and identification of bacterial strains:

Membrane filtration technique was used to detect the presence of selected pathogenic bacteria in collected water samples according to **APHA (2005).** Detection of *E. coli* O157:H7 was carried out according to **Zadik *et al*. (1993)** using HiCrome EC O157:H7 selective agar (HiMedia, India) plates. Detection of *pseudomonas aeruginosa* was carried out according to **King *et al.* (1954)** using HiFluoro *Pseudomonas agar base* selective agar (i.e. HiMedia media). Detection of *Bacillus subtilis* was carried out according to **Murray *et al.* (2003)** using HiCrome bacillus agar selective media (HiMedia, India). Detection of *Staphylococcus epidermis* was carried out according to **Baired-Parker (1962**) using Baired-Parker agar medium.

Biochemical identification of the isolated bacterial strains (*E. coli* O157:H7, *Bacillus subtilis, Pseudomonas aeruginosa and Staphylococcus epidermis*) was carried out according to **ISO (1991)** and **APHA (2005)** and confirmed by protocol A (BIOLOG GNIII containing MicroStation™ system/MicroLog Version 5.1.1., USA).

Preparation of bacterial strains and inocula:

A loopful from each stored slant of the four selected strains (*E. coli* O157:H7, *Bacillus subtilis, Pseudomonas aeruginosa and Staphylococcus epidermis*) was added to 5 ml TSB (DIFCO Co.)

tube and incubated at 37^{o}C for 24 h and then subcultured into 50 ml TSB flask incubated at 37°C for 24 h. Incubated flasks were then centrifuged at 3000 rpm for 20 min. and the pellets were separately washed three times using sterile deionized water. Each pellet was re-suspended in 10 ml sterile deionized water that was used as inoculum to avoid introducing additional nutrients or minerals to the water **(APHA, 2005)**.

Preparation of chlorine water:

Chlorine gas from Giza water treatment plant was injected into one liter brown glass- stopper bottle containing 250 ml chlorine-free distilled water and stored in dark at 4^{o}C **(APHA, 2005)**.

Determination of chlorine concentration:

Determination of initial chlorine concentration was carried out according to **APHA (2005)**. In brief, two ml H_2SO_4 (conc.), 1 gm potassium iodide (KI) and 0.5 ml chlorine water were added into 100 ml free chlorine distilled water in 250 ml conical flask with quick feet stopper. The flask was incubated in dark for 5 min then titrated with sodium thiosulfate (titrant) until the pale yellow color reached then 1 ml starch solution was added and titration continued until the blue color disappeared. Chlorine concentration (mg/Liter) was calculated from the following equation:

mgCl_2/L = ml of titrant X 0.9 (MW of Cl x N of sodium thiosulfate titrant) / ml of sample

Determination of chlorine breakpoint for the examined bacterial strains:

For each bacterial strain, nine conical flasks (250 ml each with quick fit stopper) containing 100 ml previously sterile Nile river water (by filtration via 0.2 µm pore size) were used. Each flask was then inoculated with 0.5 ml of *E. coli* O157:H7 and then inoculated with one of the eight different doses of chlorine water from (0.2, 0.6, 1.0, 1.4, 1.8, 2.2, 2.6 and 3.0 mg/L), while the remaining ninth flask was used as a control. All flasks were incubated for 10 min contact time in a dark place. After that, one ml was taken from each flask and used for determination of *E. coli* O157:H7 counts using plate count agar. The residual chlorine in each flask was determined by 5 ml phosphate buffer solution and 5 ml DPD indicator reagent. The developed color was immediately recorded using spectrophotometer at wavelength 515 nm. The breakpoint for each strain was determined by drawing the chlorine doses (mg/L) (X axis) versus residual chlorine (mg/L) (Y axis) **APHA (2005)**.

The previously mentioned steps were separately repeated with the other 3 previously prepared bacterial strains (*Bacillus subtilis, Pseudomonas aeruginosa and Staphylococcus epidermis*).

Statistical analysis:

The relationship between the residual chlorine doses and the four examined bacterial strains at different chlorine doses after contact time was explained by (R^2) using regression analysis. All the obtained data were transformed in decimal logarithms and processed by SPSS, 19. computer application.

Results

Identification of the four isolates:

The four bacterial isolates were identified up to species level after being submitted to biochemical tests (Table 1). Furthermore, identification was confirmed using BIOLOG GN III.

The four identified bacterial isolates were *E. coli* O157:H7, *Bacillus subtilis, Pseudomonas aeruginosa and Staphylococcus epidermis.*

Table 1. Morphological and biochemical characterization of the bacterial isolates according to ISO (1991) and APHA (2005).

Biochemical Tests	I	II	III	IV
Gram stain	-	+	-	+
Cell shape	Rod	Rod	Rod	Coccid
Motility	Highly motile	Motile	Motile	Non motile
Catalase	+	+	+	+
Oxidase	-	-	+	+
Indole test	+	+	-	-
Starch hydrolysis	-	+	-	-
Citrate utilization	Utilized	Utilized	Utilized	Not utilized
Vogesproskaur	+	-	-	-
Growth temperature	45**ºC**	37**ºC**	42**ºC**	45**ºC**
Growth at pH	6.8 ± 0.2	7.1 ± 0.2	7.2 ± 0.2	6.8 ± 0.3
Probable organism	***E. coli O157:H7***	***Bacillus subtilis***	***Pseudomonas aeruginosa***	***Staphylococcus epidermis***

Chlorine requirements for inactivation of *E. coli* O157:H7, *Staphylococcus epidermis, Pseudomonas aeruginosa and Bacillus subtilis*:

The initial counts of *E. coli* O157:H7, *Staphylococcus epidermis, Pseudomonas aeruginosa* and *Bacillus subtilis* were 3.3 $X10^6$, 7.2 $X10^{6,}$ 2.0 $X10^6$ and 1.2 $X10^6$ CFU/ml, respectively, before exposure to any chlorine dose.

Removal percentage of *E. coli* O157:H7 reached 99.98 % with about 4 log reduction at chlorine dose 1.4 mg/L and residual chlorine 0.31 mg/L. Log count of *E. coli* O157:H7 was reduced from 5.98 to 1.48 CFU/ml at 0.2 and 3.0 mg/L chlorine doses, respectively.

The chlorine breakpoint determined at 1.0 mg/L chlorine dose and the residual chlorine 0.24 mg/L, was the optimum chlorine dose required to give high eradication effect of *E. coli* O157:H7.

Statistical analysis showed that the regression (R^2) between the residual chlorine and *E. coli O157:H7* counts were 46 %, indicating that the increase in chlorine dose resulted in decrease of *E. coli O157:H7* counts.

Removal percentage of *Staphylococcus epidermis* reached 100 % with about 7 log reduction at chlorine dose 2.2 mg/L and residual chlorine 0.6 mg/L. Log count of *Staphylococcus epidermis* was reduced from 6.08 to 0.0 CFU/ml at 0.2 and 3.0 mg/L chlorine doses respectively.

The chlorine breakpoint determined at 2.2 mg/L chlorine dose and the residual chlorine 0.6 mg/L, was the optimum chlorine dose required to give high eradication effect of *Staphylococcus epidermis*

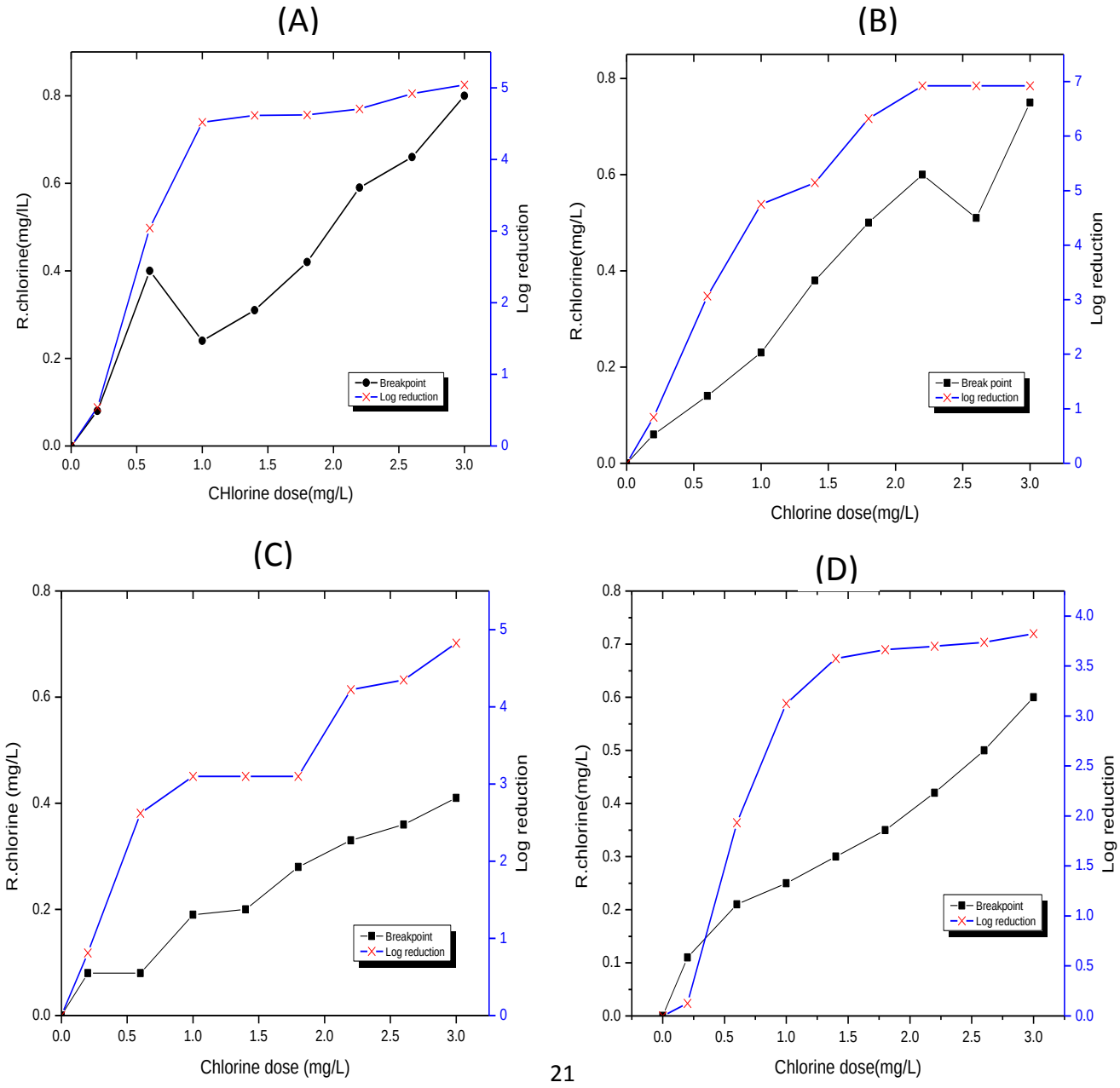
Statistical analysis showed that the regression (R^2) between the residual chlorine and *Staphylococcus epidermis* counts was 32 %, indicating inversely proportional relationship. Removal percentage of *Pseudomonas aeruginosa* reached 99.92% with about 3 log reduction at chlorine dose 1.4 mg/L and residual chlorine 0.2 mg/L. Log count of *Pseudomonas aeruginosa* was reduced from 5.49 to 1.48 CFU/ml at 0.2 and 3.0 mg/L chlorine doses respectively.

The chlorine breakpoint determined at 1.4 mg/L chlorine dose and the residual chlorine 0.2 mg/L. was the optimum chlorine dose required to produce high eradication effect of *Pseudomonas aeruginosa*.

Statistically, the increase in chlorine dose led to decrease in *Pseudomonas aeruginosa* counts, while the regression (R^2) between them was 33%.

Removal percentage of *Bacillus subtilis* reached 98.83% with about 2 log reductions at chlorine dose 0.6 mg/L and residual chlorine 0.21 mg/L. Log count of *Bacillus subtilis* was reduced from 5.95 to 2.26 CFU/ml at 0.2 and 3.0 mg/L chlorine doses, respectively. The optimum chlorine dose required to give high eradication effect of *Bacillus subtilis* was determined at 0.6 mg/L chlorine dose and the residual chlorine 0.21 mg/L. Statistically, *Bacillus subtilis* counts were inversely proportional to chlorine doses, while the regression between them reached to 33%.

Fig (4): Log reduction and residual chlorine against four bacterial isolates. (A) *E. coli O157:H7*, (B) *Staphylococcus epidermis*, (C) *Pseudomonas aeruginosa* and (D) *Bacillus subtilis*



Discussion

Microbial contamination of drinking-water contributes to disease outbreaks and background rates of disease in developed and developing countries worldwide. Control of waterborne disease is an important element of public health policy and an objective of water suppliers. Chlorine is one of the most favorable used water disinfectants. Chlorine gas and water react to form hypochloric acid HOCl and hydrochloric acid (HCl). In turn, the HOCl dissociates into hypochlorite ion (OCl^-) and hydrogen ion (H^+) **(LeChevallier and Au, 2004).**

In the present work, it was found that the *Staphylococcus epidermis* and *Pseudomonas aeruginosa* were more resistant to chlorine than *E. coli* O157:H7 and *Bacillus subtilis.* Although efficiency of a disinfectant is strongly affected by organism-dependent characteristics, it depends to a large extent on environmental factors such as pH, temperature or the availability of the active disinfecting species modulated by the presence of interfering compounds **(Morato *et al.*, 2003)**. In addition to that and when submitted to disinfection treatments, *E. coli* can rapidly enter a viable but non-culturable (VBNC) state, rendering it difficult to detect by classical culture methods **(Grey and Steck, 2001; Villarino *et al.*, 2003).**

The mechanism by which bacteria acquire resistance to chlorine and its derivatives is not well understood. It is known that environmental conditions (e.g., temperature) can diminish resistance to stress factors such as chlorine **(Wu *et al.*, 2007).** The phenomenon of indirect resistance occurs when additive environmental and stress factors cause the expression of genes that increase bacterial resistance (**Berry *et al.*, 2010)**. Additionally, suspended solid particles and organic matter can provide protection to microorganisms by generating a demand for residual chlorine, which decreases the availability of chlorine and weakens the disinfection process. Microbial aggregation is another factor that confers resistance to chlorine disinfection (**Virto *et al.*, 2005; Winward *et al.*, 2008)**.

In the present study, the removal percentage of *E. coli* O157:H7 reached 99.98 % with about 4 log reduction at chlorine dose 1.4 mg/L and residual chlorine 0.31 mg/L. An EPA report noted that inactivation rates of *E. coli* O157:H7 and wild-type *E. coli* were similar **(Rice and Johnson, 2000)**. Water utilities in the USA maintain a median chlorine residual of 1.1 mg/L, with a 45 min. before the first point of use in the distribution system. *E. coli* O157:H7 is therefore unlikely to

survive conventional water treatment practices. However, the possibility of acquiring STEC infection from drinking water remains. For example, not all municipal water utilities use chlorine, and adverse conditions can greatly diminish chlorine levels. Weather conditions may also degrade drinking water quality. *E. coli* O157:H7 is not resistant to chlorination practices commonly used to purify water **(Rice and Johnson, 2000)**. Moreover, it was found that *E. coli* O157:H7 isolated from environment and treated with chlorine at concentrations of 0.25 and 0.50 mg/ml showed greater resistance than two reference strains when treated with 0.50,0.25 and 0.10 mg/ml chlorine for 1, 5 and 10 min, respectively **(Ryu and Beuchat, 2005)**. Chlorine concentrations of 0.2 mg/L for 3 min and 0.5 mg/L for 6 min effectively caused 99.99% reduction of *Escherichia coli* and 99% reduction of *Salmonella typhi*, respectively **(Ram and Malley, 1984; Fass *et al.*, 1996**). Other workers mentioned that 1.4 mg/L of chlorine greatly affected the growth of total coliform where the lethal results were 100% in some samples and 96.6% in other samples. They also added that the chlorine dose up to 1.6 mg/L caused appreciable injury (94.3%) of *E. coli* population (lethality from 48.2 to 85 %) after 10 min contact time (**McFeters *et al*., 1986)**.

In the present study, the removal percentage of *Staphylococcus epidermis* reached 100 % with about 7 log reductions at chlorine dose 2.2 mg/L and residual chlorine 0.6 mg/L. Other workers noticed that free chlorine residuals of 0.05, 0.1, 0.2 and 0.4 mg/L for 3.4 to 5.2, 2.8 to 4.0, 1.7 to 3.0 and 0.8 to 2.0 min, respectively, have been used for disinfecting drinking water with 99.99 % reduction on fecal streptococci **(Wiedenmann *et al*., 1997)**.

In the present study, the removal percentage of *Pseudomonas aeruginosa* reached 99.92% with about 3 log reduction at chlorine dose 1.4 mg/L and residual chlorine 0.2 mg/L. **Carson *et al.* (1972)** have reported that *Pseudomonas aeruginosa* growing in distilled water was markedly more resistant to acetic acid, glutaraldehyde, chlorine dioxide, and a quaternary ammonium compound than were cells cultured on tryptic soy agar.

In the present work, the removal percentage of *Bacillus subtilis* reached 98.83% with about 2 log reductions at chlorine dose 0.6 mg/L and residual chlorine 0.21 mg/L. Other workers showed that the inactivation process of *Bacillus subtilis* spores with chlorine was characterized by a lag phase and a log phase of inactivation. Since, the rate of inactivation would be increased at either higher disinfectant concentration or longer reaction time. The ability of chlorine inactivating spores was stronger under acidic condition than that in alkali condition. It has been also indicated that

Bacillus subtilis spores are more resistant to chlorine than *Bacillus anthracis* spores (**Liu *et al.*, 2013)**.

Other studies have shown that chlorine rinses can decrease the bacterial load by values ranging from 1 log CFU/g to 3.15 logs CFU/g **(Beuchat *et al.*, 2004; Bruchet and Duguet, 2004; Nthenge *et al.*, 2007),** depending on inoculation method, chlorine concentration, contact time, and the target bacteria tested. There are high possibilities that chlorine-resistant bacterial strains may have biotechnological applications for the production of extremely resistant enzymes (**Martins, *et al.*, 2013)**.

The effect of relatively high concentrations of hypochlorite (at concentrations normally used in hospitals for the decontamination of tissues and surfaces) on both *L. pneumophila* and *Escherichia coli* was investigated. The obtained data suggested that *Legionella* spp. might be somewhat more resistant to these high chlorine concentrations than are the coliform bacteria. They also raised the suspicion that the amount of residual chlorine recommended for standard water purification might not be sufficient for killing *L. pneumophila* when the bacteria are present in high numbers **(Wang and Ahearn., 1997)**.

Molecular mechanisms confer resistance of some bacteria to chlorine through the expression of certain genes in response to stress factors such as oxidizing agents, variations in temperature, osmotic shock or small amounts of organic matter present in the medium. Such growth conditions could presumably alter the bacterial inactivation process by reducing bacterial metabolism or by changing the permeability of the cell membrane **(Berry *et al.*, 2010).**

In the present work, the bactericidal effect of chlorine was tested in sterilized Nile water to simulate the same environmental condition from which the examined bacteria were isolated. Other workers concluded that it would be useful to test different inactivation conditions on distinct groups of opportunistic and pathogenic bacterial species that are phylogenetically related to each other and to address the impact of organic matter content on the efficiency of chlorine disinfection for these groups of species **(Martínez-Hernández *et al.*, 2013)**.

Generally, it can be concluded that the breakpoint of *E. coli* O157:H7, *Bacillus subtilis, Pseudomonas aeruginosa* and *Staphylococcus epidermis* were recorded at chlorine doses 1, 0.6, 1.4 and 2.2 mg/L, respectively. The bacterial strains isolated from Nile water in the present work

represent a serious health risk because they are mostly of related to species and genera that include opportunistic and pathogenic microorganisms. With the exception of *E. coli* 0l57H: 7, the other tested strains (*Staphylococcus epidermis*, *Pseudomonas aeruginosa* and *Bacillus subtilis*) are non-fecal in origin and are different from coliforms. Thus, there is an urgent need to improve water regulations to include species other than the traditional indicators of water quality.

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