Efficiency of primary chlorination, clarification and final disinfection on *Pseudomonas aeruginosa* under laboratory conditions in raw water

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ABSTRACT

In order to inactivate *Pseudomonas aeruginosa* in raw waters, we investigate the efficiency of primary chlorination alone and combined chlorination and clarification in surface water naturally containing *P. aeruginosa*. Final disinfection was performed in clarified surface water and groundwater both contaminated with *P. aeruginosa*. The results obtained under laboratory conditions show that coagulant aluminum sulfate (AS) and cationic polymer polyelectrolyte contribute significantly to reducing turbidity with the inactivation of cultivable cells of *P. aeruginosa* in different water samples. Our findings also suggest that while chlorination may be a satisfactory method for controlling and preventing *P. aeruginosa* growth in water intended for human consumption, it does not eradicate all cultivable bacteria in raw surface water. *P. aeruginosa* was nonetheless shown to be relatively sensitive to sodium hypochlorite, and the effectiveness of chlorine was greater in natural groundwater than in clarified surface water.

Key words | chlorine demand, free residual chlorine, groundwater, jar-test, *P. aeruginosa*, surface water

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INTRODUCTION

Disinfection, a chemical process used to control disease-causing microorganisms by killing or inactivating them, is unquestionably the most important step in drinking water treatment (WHO 2006). Thus, disinfection represents one of the greatest advances in public health protection (Charrois & Hrudey 2007). *P. aeruginosa* is recognized as the cause of hospital-acquired infections with potentially serious complications and is resistant to many antibiotics (Kallel *et al.* 2008). It can multiply in aquatic environments and also on the surface of suitable organic materials in contact with water (Whitchurch *et al.* 2005; WHO 2006). At the beginning of the 20th century, chlorine was introduced into the urban water supply to improve its hygienic quality by eliminating waterborne bacterial pathogens and the subsequent transmission of waterborne diseases (Galal-Gorchev 1996; Nieuwenhuijsen *et al.* 2000). In fact, chlorine alleviates many disagreeable tastes and odors, acts as a potent germicide, and eliminates bacteria able to form biofilms that commonly grow in water reservoirs (Cozad & Rhonda 2003). Moreover, turbidity in water is caused by suspended and colloidal matter that affects the efficiency of disinfection with chlorine (Galal-Gorchev 1996). Thus, colloids can be removed by clarification processes that include coagulation, flocculation, decantation, and filtration (Bouyer *et al.* 2005; Koohestanian *et al.* 2008). Surface waters contain obligate pathogens as well as opportunistic pathogens, such as *P. aeruginosa*. By its presence throughout the environment, *P. aeruginosa* may lead to contamination of several drinking water points used by humans (Ramsey &

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Wozniak 2005; Whitchurch *et al.* 2005). Maintenance of the microbiological quality of water has been an important means of preventing waterborne diseases throughout the 20th century (Suman *et al.* 2008). So water treatment processes must transform raw water into safe drinking water.

The Tunisian government, represented by the National Society of Exploitation and Distribution of Water (SONEDE) afford great importance to the quality of water. The surface waters of Medjerda River show high loads of *P. aeruginosa* (10^3 cfu/100 mL) and sometimes this bacterium may be found in groundwater in Kairouan region. In this context, it is important to assess whether the present chlorination system employed in Tunisian water plant is sufficient to inactivate *P. aeruginosa* growth in water. Laboratory experiments were performed to show the effect of sodium hypochlorite as a disinfectant agent in the inactivation of *P. aeruginosa* during the primary chlorination, clarification, and final disinfection of different raw waters sources intended for human consumption in Tunisia.

MATERIALS AND METHODS

Origin of the collected samples

Three different water samples were used in this study. Two samples of raw surface water (SW) were collected from the Medjerda River in Tunisia. The first was an untreated surface water sample (SW1) harvested at the entry point of the Belli water treatment plant located in the Cap-Bon region, the second was a clarified water (SW2) sample collected from the same source after a filtration through a sand filter (AQUAZUR type T), and the third was a sample of groundwater (GW) collected from the Kairouan region in Tunisia.

Physicochemical analysis

Physicochemical analyses were conducted within 12 hours of sample collection and included turbidity measurements which were performed at 25°C using a turbidimeter (2100 N-Hach). The measured turbidity was expressed in nephelometric turbidity units (NTU). Water salinity was determined using a salinimeter (Myron LDS Meter, USA) and the total dissolved salt measurements were expressed in mg/L. Finally, the pH of each water sample was measured with a pH meter (Metrohm 744, Switzerland). All of the measurements were done in triplicate and their averages were calculated.

Determination of the cultivable *P. aeruginosa* in the water samples

The quantification of *P. aeruginosa* in the different water samples were conducted as following: a volume of 250 mL of each water sample was collected and free chlorine was neutralized by adding sodium thiosulfate. A volume of 100 mL was filtered by membrane filtration (Sartorius AG, Goettingen, Germany) through a 0.45 μm pore size cellulose-ester membrane (Sartorius AG 37070). The membrane was then placed on cetrimide agar (Pronadisa, Spain) and was incubated at 37°C for 48 h. Following each culture period, suspected *P. aeruginosa* colonies that showed the characteristic appearance and color on cetrimide agar plates were examined. Several phenotypic tests were used for the identification: gram-negative, oxidase-positive rods, growth at 42°C in nutrient broth (Bio-Rad, France), and the ability to produce pyocyanin and pyoverdin pigments on King A (Pronadisa, Spain) and on King B (Pronadisa, Spain) media respectively. Typical colonies that grew at 42°C and produced pyocyanin and pyoverdin pigments were enumerated as *P. aeruginosa*.

Effect of chlorine on *P. aeruginosa* inactivation

Chlorine solution preparation

A volume of 10.4 mL was taken from a sodium hypochlorite solution at 30.3°F (French chlorometric degree) and was added to 989.6 mL of distilled water to render a stock chlorine solution at concentration of 1 g/L of free chlorine. Various chlorine solutions concentrations were prepared daily by diluting the stock solution of 1 g/L sodium hypochlorite in sterile deionized water for subsequent use. All experiments were conducted at room temperature at 25°C.

Chlorine demand assays

The chlorine demand experiments were performed for the three water samples, namely the untreated surface water (SW1), the clarified surface water (SW2), and the groundwater (GW), that naturally contain different concentrations of

P. aeruginosa (Table 1). A series of 13 bottles, each containing 1 L of water from SW1 were supplemented with increasing chlorine solutions at concentrations: 0, 2, 2.2, 2.4, 2.6, 2.8, 3, 3.4, 3.6, 3.8, 4, 4.2, and 4.6 mg/L as a pre-oxidation step. This step represented the primary chlorination. Following a contact time of 1 h in the presence of the chlorine solutions (LeChevallier *et al*. 1981; Rizzo *et al*. 2008), sodium thiosulfate was added in order to neutralize residual chlorine. Then, *P. aeruginosa* colonies were counted by means of a membrane filtration method as described above. To determine the chlorine demand for SW2 and GW, the following doses of chlorine were tested: 1, 1.5, 2, 3, 3.5, 4, 5, 6, 7, and 8 mg/L. After, 1 h of contact time with chlorine solutions, the free residual chlorine concentrations were verified in duplicate. The experiments were performed at temperature of 25°C.

Measurement of the free residual chlorine

The free chlorine concentrations were determined using the N-diethyl-p-phenylenediamine (DPD) colorimetric method as previously reported by Harp (2002). DPD free chlorine reagent powder for 5 mL samples were provided by Permachem® Reagents (Hach Co., DPD Free chlorine Powder Pillows Cat. USA). Color intensity was measured by means of a comparator apparatus (Hach, Model CN-66 F, USA) which determined the concentration of free and total chlorine range (0–3.5 mg/L).

Effect of the primary chlorination on P. aeruginosa

In this study we chose the bacterium *P. aeruginosa* ATCC 27853 purchased from the Charles Nicole Hospital in Tunis in order to contaminate raw water samples (SW2 and GW).

Forty litres of raw water was first collected from surface water (SW1), followed by a study to determine the break point chlorination by measuring free residual chlorine. Thereafter, in the primary chlorination step, SW1 contained naturally *P. aeruginosa* at concentration of 10^3 cfu/100 mL. The water was treated for 1 h with chlorine solutions, then residual chlorine was neutralized and *P. aeruginosa* was subsequently collected, grown, and enumerated. The experiment was performed at 25°C.

Jar testing

Jar tests were used to determine the optimal dose of coagulant aluminum sulfate (AS) [$(Al_2(SO_4)_3.18\ H_2O)$] and cationic polymer (polyelectrolyte) required for flocculation. The jar testing experiments were performed in a programmable jar test apparatus (Aqualytic flocculator, Germany) as follows: 1 L of untreated surface water (SW1) was placed in multiple glass beakers then water samples were mixed by shaking at 100 rpm/30 s. Various amounts AS were subsequently added to each beaker (0, 5, 10, 15, 20, 25 and 30 mg/L). The beakers were mixed at 100 rpm for 1 min, followed by slow mixing at 40 rpm for 30 min and then settled 0 rpm for 30 min. The same experiment was performed with a cationic polymer at various doses (0, 5, 10, 15, 20, 25, and 30 μg/L) to determine the optimal dose for removal of turbidity. After 30 min of settling, the turbidity of each jar was measured by means of a turbidimeter (N-Hach). The lowest dose less than 5 NTU was maintained as the optimal dose of coagulant or cationic polymer tested.

Combined action of primary chlorination and conventional clarification

At first SW1 naturally containing *P. aeruginosa* at a level of 10^3 cfu/100 mL was supplemented with chlorine at various doses: 2, 3.2, 3.4, 4, and 4.6 mg/L. The optimum dose of coagulant AS and cationic polymer obtained by jar testing was successively added to each glass beaker and settled for

Table 1 | Physicochemical characteristics with the concentration of *P. aeruginosa* of raw water sources

	Samples	pH	Turbidity (NTU)	TDS (mg/L)	*P. aeruginosa* concentration cfu/100 mL
Surface water (Medjerda river)	SW1	7.8	10	1400	1×10^3
	SW2	7.6	0.3	2000	4
Groundwater (Kairouan region)	GW	7.9	0.18	1100	0

GW: groundwater, SW1: untreated surface water; SW2: clarified surface water; NTU: nephelometric turbidity unit; TDS: total dissolved salts. All measured values are averages.

30 min. Then, 250 mL of supernatants was collected from SW1 sample and filtered through sterile Whatman paper in sterile bottles, and cultivable *P. aeruginosa* were enumerated in 100 mL of filtered water. The experiment was conducted at room temperature at 25°C.

Effect of chlorine in the final disinfection

The demand of chlorine for the two water samples SW2 and GW was determined, prior to contamination by *P. aeruginosa*. Following 1 h of contact time with chlorine, the obtained chlorine dose gave the following values of free residual chlorine: 0.5, 1, 1.5, and 3 mg/L. The next step was to contaminate SW2 and GW with *P. aeruginosa* ATCC 27853 at three different final concentrations. Twelve litres of water samples (SW2 and GW) were contaminated by *P. aeruginosa* at concentrations of 10^5, 10^3, and 100 cfu/100 mL, respectively. In order to inactivate *P. aeruginosa* in the SW2 and GW samples, chlorine was used for 1 h of contact time at concentrations of: 1.5, 2, 3, and 5 mg/L for SW2 and 1, 1.5, 3, and 5 mg/L for GW; then the *P. aeruginosa* cfu and the amount of free residual chlorine in the water samples were determined. One experiment was performed at room temperature 25°C with duplicate determinations of free residual chlorine and *P. aeruginosa* cfu.

RESULTS

Primary chlorination

Our experiments were performed in the laboratory in order to simulate the efficiency of different steps of water treatment in the inactivation of *P. aeruginosa*. The physicochemical characteristics of the untreated surface water (SW1) are shown in Table 1 with the average concentration of *P. aeruginosa* in each water sample tested. SW1 displayed turbidity >5 NTU, while SW2 and GW both recorded relatively low turbidity levels<1 NTU, with a pH<8; no adjustments were made to the pH of the samples. The chlorine demand curve (Figure 1) showed a typical break point chlorination of sample SW1 obtained at a chlorine dose of 3.4 mg/L which was sufficient to oxidize all of the organic matter in this sample, while a dose of chlorine greater than 3.4 mg/L recorded a suitable

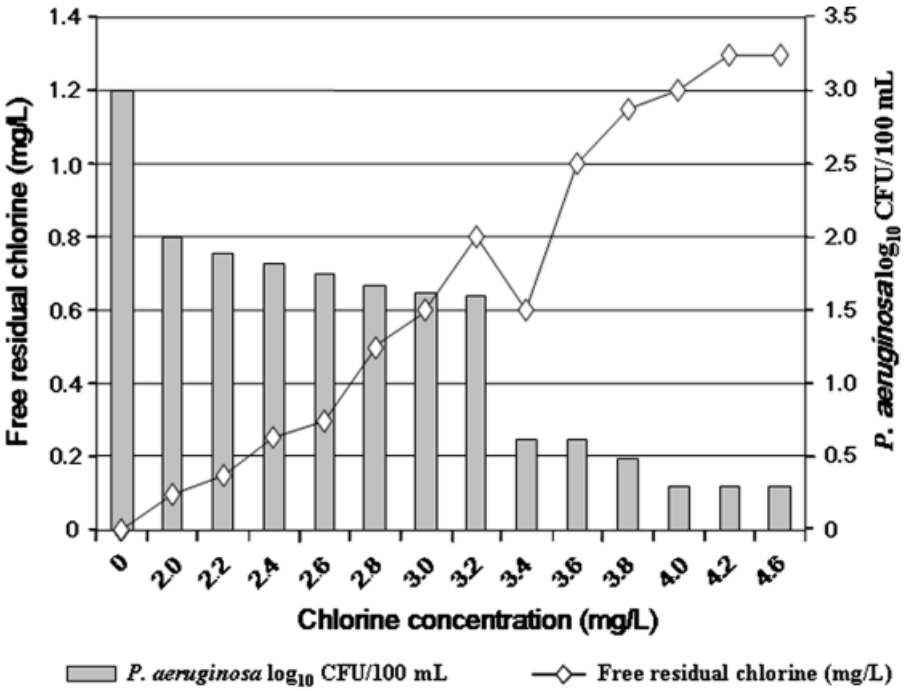

Figure 1 | Effect of primary chlorination on untreated surface water (SW1) naturally containing *P. aeruginosa* during one hour of contact time. This figure shows a typical break point chlorination curve (◇) obtained after chlorine dose added on SW1 and in parallel cultivable *P.aeruginosa* were enumerated after one hour of contact time, in order to show the effect of disinfectant chlorine. Values are the average of duplicate measurements.

free residual chlorine level of approximately 1 mg/L. Our results show an interesting decrease of *P. aeruginosa* from 3 $\log_{10}$ to 0.5 $\log_{10}$ cfu/100 mL following treatment with chlorine. Meanwhile, *P. aeruginosa* remained cultivable at a level up to 0.5 $\log_{10}$ cfu/100 mL after 1 h of contact time with the chlorine (Figure 1). Chlorine used as an oxidizing agent for primary chlorination was ineffective in completely inactivating *P. aeruginosa* in SW1 at a level of 10^3 cfu/100 mL. Furthermore, *P. aeruginosa* resisted at a concentration of 1.3 mg/L of free residual chlorine and persisted in a cultivable form in SW1.

Combined action of primary chlorination and clarification

The first step was to determine the optimum doses of coagulant (AS) and flocculant (cationic polymer) by means of a jar test for SW1. The required doses of AS and of cationic polymer were selected to obtain a turbidity of less than 5 NTU, as recommended by the WHO (2006).

The optimum dose of AS was 10 mg/L and the turbidity measured was 3.82 NTU, corresponding to a removal turbidity of 56.3%. Indeed, by doubling the amount of coagulant, the percentage of turbidity increased by 12.1% (Figure 2).

The optimum amount of cationic polymer was 10 μg/L, resulting in 2.65 NTU and corresponding to 30.62% of removal turbidity (Figure 3). Results of the combined effect of primary chlorination and clarification on the water sample (SW2) contaminated by *P. aeruginosa* showed the bacterial count dropped from 10^3 cfu/100 mL to 0 cfu/100 mL at the effective chlorine dose of 4 mg/L, with free residual chlorine at 1.1 mg/L (Table 2).

Effect of chlorine in *P. aeruginosa* inactivation from clarified surface water

Clarified surface water was contaminated with high levels of *P. aeruginosa* ATCC 27853 (10^5, 10^3, and 100 cfu/100 mL). The SW2 sample was purchased from the Belli treatment plant, after clarification. A chlorine dose of 5 mg/L was ineffective to inactivate completely *P. aeruginosa* at a concentration of 10^5 cfu/100 mL in SW2; bacterial cells remained cultivable at a level of 2.8 mg/L of free residual chlorine. With decreasing bacterial levels, the concentrations

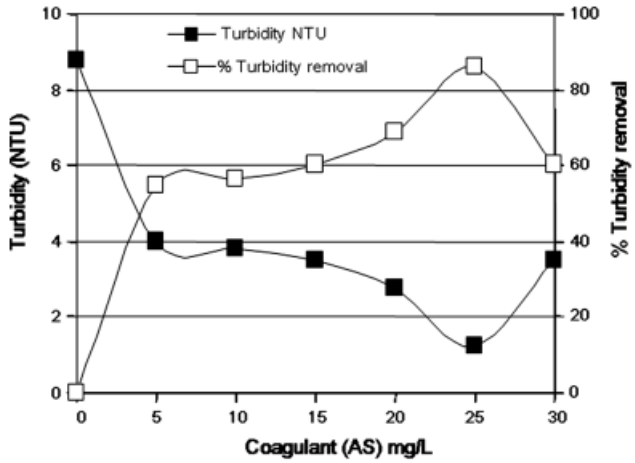

Figure 2 | Optimal doses of coagulant aluminium sulfate (AS) required by the untreated surfacewater (SW1) to remove turbidity were 5, 10, 15, 20 and 25 mg/L as determined by Jar-test. Values are the average of duplicate measurements.

of free residual chlorine increased in parallel (Table 3). At *P. aeruginosa* concentration of 10^3 cfu/100, 3 mg/L of chlorine was effective in inactivating cultivable cells with a free residual chlorine dose of 1.5 mg/L; for the weak initial contaminant of 100 cfu/100 mL, effectiveness was observed at a chlorine dose of 2 mg/L.

Effect of chlorine in *P. aeruginosa* inactivation from the groundwater

In order to inactivate *P. aeruginosa* ATCC 27853 in groundwater at 10^5 cfu/100 mL, 5 mg/L of chlorine was injected. At

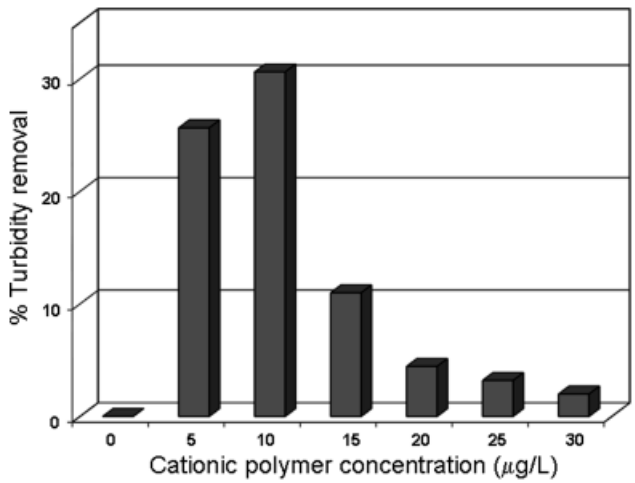

Figure 3 | Optimal dose of cationic polymer (polyelectrolyte) required by the sample water SW1 is obtained at 10 μg/L which was determined by Jar-test. Values are the average of duplicate measurements.

Table 2 | Combined effects of primary chlorination and clarification with optimal doses of aluminium sulfate (AS) and cationic polymer on survival of *P. aeruginosa* in surface water (SW1)

Parameters of samples	T	T2	T3	T4	T5	T6
Chlorine dose (mg/L)	0	2	3.2	3.4	4	4.6
Free residual chlorine[a] (mg/L)	0	0	0.7	0.5	1.1	1.3
AS dose[b] (mg/L)			10			
Cationic polymer dose[b] (μg/L)			10			
P. aeruginosa $\log_{10}$cfu/100 mL	3	2	1.3	1	–	–

T: control sample, T2, T3, T4, T5, and T6: designation of samples. [a]measured after 1 h of contact time; [b]optimal doses determined by Jar-Test; cfu: colony forming units, -: no cultivable colony. Values are means of duplicate determinations.

this step, the bacterium was cultivable at concentration of 1 cfu/100 mL in GW, accompanied by an excessive free residual chlorine rate of 1.9 mg/L, as indicated in Table 4. For an initial concentration 10^3 cfu/100 mL total inactivation was observed with a chlorine dose of 5 mg/L, in spite of a high level of residual chlorine (2.5 mg/L). For the concentration of 100 cfu/100 mL, 1.5 mg/L of chlorine was effective in inactivating *P. aeruginosa* with an acceptable level (1.1 mg/L) of free residual chlorine. Total inactivation of *P. aeruginosa* ATCC 27853 was achieved from clarified sample SW2 and from the GW with chlorine doses of 2 mg/L and 1 mg/L, respectively (Tables 3 and 4). The chlorine doses tested as a disinfected agent was more effective in inactivating *P. aeruginosa* ATCC 27853 cells from groundwater than from clarified water.

DISCUSSION

Chlorine is widely used in public drinking water plants in Tunisia in order to provide a disinfection barrier. As stated by the WHO (2006), *P. aeruginosa* is a bacterium that is sensitive to chlorine. Our results confirm that its sensitivity varies with water quality. Thus, changing water quality parameters may influence the effectiveness level of the disinfection method. In our study, the raw water samples had a pH less than 8.0, as recommended by the WHO (2006) for effective chlorine disinfection, indeed pH ranging between 6.5 amd 8.5 indicates that chlorine introduced in water will be in two forms hypochlorus acid (HOCl), which shows a bactericidal activity and hypochlorite ion (OCl^-) (Harp 2002; Wang *et al.* 2007). The persistence of *P. aeruginosa* after the primary chlorination of SW1 (Figure 1) may be explained by several factors: water turbidity, chlorine concentration, and contact time (Galal-Gorchev 1996; Hu *et al.* 1999). The presence of *P. aeruginosa* in the surface water treated with chlorine may be a consequence of a high level of turbidity which may have modulated the effectiveness of disinfection by chlorine. Indeed, LeChevallier *et al.* (1981) showed a negative correlation between turbidity and the decrease of survival coliforms in chlorinated water. Furthermore, according to WHO (2006), colloids and small particles can not only

Table 3 | Effect of disinfection by chlorine on *P. aeruginosa* ATCC 27853 survival, in clarified surface water (SW2)

		Chlorine dose (mg/L)			
Averages calculated[a]	Initial concentrations	1.5	2	3	5
Free residual Cl_2mg /L	0	0.5	1	1.5	2.8
P. a cfu/100 mL	10^5	10^4	10^3	104	32
Free residual Cl_2mg/L	0	0.7	1.2	1.5	3.5
P. a cfu/100 mL	10^3	750	80	0	0
Free residual Cl_2mg/L	0	0.7	1.2	1.7	3.5
P. a cfu/100 mL	100	1	0	0	0

P. a cfu/100 mL: Pseudomonas aeruginosa colony forming units per 100 mL.
[a]Measured after one hour of contact time. Values are means of duplicate determinations.

Table 4 | Effect of disinfection by chlorine *P. aeruginosa* ATCC 27853, survival in groundwater

		Chlorine dose (mg/L)			
Averages calculated[a]	**Initial concentrations**	**1**	**1.5**	**3**	**5**
Free residual Cl_2mg /L	0	0.4	0.8	1.5	1.9
P. a cfu/100 mL	10^5	10^3	100	30	1
Free residual Cl_2mg/L	0	0.8	0.9	1.9	2.5
P. a cfu/100 mL	10^3	80	10	1	0
Free residual Cl_2mg/L	0	0.6	1.1	2.2	2.5
P. a cfu/100 mL	100	0	0	0	0

P. a cfu/100 mL: Pseudomonas aeruginosa colony forming units per 100 mL.
[a]Measured after one hour of contact time. Values are means of duplicate determinations.

protect microorganisms from the effect of disinfectants but also stimulate bacterial growth. To show the impact of turbidity on disinfection level, optimal doses of coagulant aluminum sulfate and cationic polymer flocculant agents were added to the SW1 sample following primary chlorination. Aluminum sulfate was chosen because it is a commonly used coagulant and has been shown to be less toxic than ferric chloride and chitosan against *Daphnia magna* (Rizzo *et al.* 2008). Our results indicate that clarification is a key step in the inactivation of *P. aeruginosa* following the primary chlorination treatment process. In fact the concentration of cultivable *P. aeruginosa* decreases from 1.1 $\log_{10}$cfu/100 mL (Figure 1) to 0.6 $\log_{10}$cfu/100 mL with the same dose (3.4 mg/L) of chlorine (Table 2). This is in agreement with a previous study by Medema *et al.* (1998) who showed that in the absence of a chemical coagulant, microbe inactivation was low because sedimentation velocities were in fact low. However, the clarification step without primary chlorination was incapable of inactivating cultivable cells of *P. aeruginosa* from SW1 sample (Table 2).

The values of free residual chlorine shown in Tables 2, 3, and 4 were relatively high, as recommended by the WHO (2006), yet remain acceptable in extreme cases for good bacteriological quality. Thus, the choice of 1.2 mg/L for free residual drinking water chlorine as recommended by the National Society of Exploitation and Distribution of Water (SONEDE) in Tunisia proves to be suitable in order to protect water, in the event of accidental contamination by *P. aeruginosa*. Our results demonstrate that the use of chlorine to disinfect drinking water did in fact inactivate the culturability of *P. aeruginosa.* This confirms previously reported studies (Medema *et al.* 1998). We also showed that at a high concentration of bacteria, the chlorine agent was unable to inactivate all of the bacterial cells, which suggests that *P. aeruginosa* may be resistant to chlorine during the final disinfection at high-level contamination. Effectiveness may also be hindered by an aggregation of microbial cells and nutrient limitations are common conditions in potable water and thus may contribute to the persistence of disinfectant-resistant organisms (Barbeau *et al.* 2005; Shi *et al.* 2009). Nevertheless, maintaining a residual disinfectant concentration after a given period may prevent bacterial regrowth (Laplace *et al.* 1997; WHO 2006). Moreover, several studies show the implication of *P. aeruginosa* in the formation of the biofilms in drinking water distribution systems (Batté *et al.* 2003; Bressler *et al.* 2009). According to Harp (2002), free residual chlorine is adjusted to maintain a minimum level of 0.2 mg/L throughout the distribution system and the presence of free residual chlorine is an indicator of adequate disinfection. Indeed, SONEDE in Tunisia permits a free residual chlorine rate ranging between 0.8 and 1.2 mg/L in water prior to storing it in reservoir tanks in order to protect treated water against re-contamination from the point of chlorination to the point of use during its travel throughout the distribution system.

CONCLUSIONS

Chlorine, used in primary chlorination as an oxidizing and disinfection agent, is capable of inactivating the pathogenic opportunist *P. aeruginosa* from raw water, and while the

clarification step enhances the chlorine's effectiveness, the combined effect of primary chlorination and clarification are more efficient to inactivate the bacteria from water. Turbid water should thus be clarified wherever possible to enable chlorine to be more effective.

A higher level of contamination could compromise the efficacy of the disinfection process by reducing its effective dose. Thus it may be advisable to inject multiple low doses of chlorine in highly *P. aeruginosa* contaminated water for better disinfection.

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