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Inactivation kinetics of gram-negative bacteria in the presence of residual free chlorine

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ABSTRACT

Inactivation kinetics of six chlorine tolerant gram-negative bacterial strains (n = 6) were studied in a water distribution system, fed with chlorinated drinking water (0.5–1 mg/L residual free chlorine (RFC)), at a hydraulic retention time of 120 min. These strains were persistently isolated after chlorination that interestingly led to further examine their inactivation kinetics *in-vitro*. The data were fit into Chick–Watson's first-order kinetics model to find decay rate '*k*', CT factor, 2 log reduction ($T_{99\%}$) and 3 log reductions ($T_{99.9\%}$) of the isolates. All the six strains were tolerant to 0.5–1 mg/L RFC at a contact time of 30 min. *Morganella morganii* subsp. *sibonii*, *Proteus myxofaciens*, *Enterobacter* and *Pseudomonas aeruginosa* showed the lowest inactivation at RFC 3.5 mg/L for 30 min. *Providencia rustigianii* (R^2 = 0.43 ± 0.02) was the most tolerant out of all the six isolates, 2 log inactivation of *Providencia rustigianii* was achieved after 180 min with 5 mg/L RFC. *Morganella morganii* subsp. *morganii* was somewhat sensitive to RFC; log reduction was achieved within 30 min at 1.5 mg/L RFC. Unsatisfactory disinfection using low-level free chlorine (0.5–1 mg/L RFC at 30 min), harbors potentially tolerant pathogenic strains in a water system.

Keywords: Inactivation kinetics; Chick–Watson model; Chlorine inactivation; Gram-negative bacteria; CT factor; Log reduction; Decay rate; Public health

1. Introduction

Water should not only be aesthetically clean but free of waterborne pathogens. An adequate supply of safe drinking water and sanitation is significant, otherwise may perhaps have negative health impacts like diarrhea [1,2]. A safe water system comprises a suitable disinfection method and ensures safe storage of treated water. Chlorination using sodium or calcium hypochlorite is one of the most effective modes of disinfection of drinking water [3]. Chlorine is an antimicrobial substance with a strong oxidizing ability [4]. When chlorine is added to water, molecules of hypochlorite (ClO^-) and hypochlorous acid (HClO) are produced that reacts with inorganic and organic impurities present in water and thus not available for disinfection of pathogens. Once the chlorine demand is met, breakpoint chlorination is done for the liberation of residual free chlorine (RFC) that ensures the inactivation of disease-causing organisms. The mechanism of chlorine action in pathogens may involve (1) alteration of active sites of the cellular enzymes (2) disruption of the plasma membrane (3) disparity of biological functions of proteins, and (4) deleterious effects on DNA [2–4]. Few species of gram-negative pathogens including some coliforms were found in otherwise high-quality drinking water containing good residual chlorine content. Water supply systems can not rely on the maintenance of high RFC levels in water (<2.0 mg/L) for the prevention of waterborne diseases, as it may lead to public health issues [2,3].

Several authors have suggested a number of mechanisms by which micro-organisms may become resistant to,

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or protected from disinfection. Few such mechanisms are encapsulation, growth conditions and antecedent growth environment. In addition, chlorine residual can dissipate under adverse conditions, and exposure to sunlight or organic chlorine-demand substances can greatly diminish chlorine levels. These considerations are particularly important in determining the efficacy of chlorination in a water treatment system.

The efficacy of disinfection using chlorine is dependent on the presence of a number of pathogens, pH and temperature of the water. Effective disinfection using chlorine requires an appropriate disinfection plan to ensure the protection of water [5–7]. Chlorine efficiently inactivates pathogens that are responsible for causing water-borne diseases. The effectiveness of chlorine against disease-causing pathogen is defined by the CT factor (concentration of chlorine in mg/L or ppm required for inactivation of the pathogen within a given time). CT measures chlorine inactivation of different pathogens that ensure the successful disinfection and effectiveness of a water treatment system [7–10].

The decay or inactivation rate stringently follows first-order kinetics, with respect to the concentration of both available chlorine and bacterial density. Pathogens exhibit a pattern of decay that exhibits first-order inactivation kinetics. The available chlorine disinfection kinetic model takes the first-order decay constant into consideration. A CT table was developed for some clinically significant waterborne pathogens for suggesting conditions essential for a 2 log10 (99%) or 3 log10 (99.9%) inactivation [11–14]. Chlorination has proven to be a huge success in water treatment despite different species of bacteria that differ in sensitivities towards chlorine action [15,16]. A number of mechanisms by which micro-organisms may become resistant to chlorine disinfection have been illustrated by authors. Starvation, aggregation, encapsulation, attachment to surfaces are few growth conditions that help the pathogens to survive in the presence of disinfectants [17–19]. High turbidity of water may not also favor the action of RFC on the bacterial cells. Chick–Watson's first order kinetics approach was considered in this study as it is an important parameter to define microbial resistance to disinfection [20–23]. This model of inactivation kinetics is based on the first-order reaction to finding decay rate, a significant approach for evaluation of microbial inactivation [24–27].

The removal efficiency by the chlorine concentrations was expressed as ln reduction of the bacterial population as described by [12–14].

$$\mathrm{LR} = \ln\left(\frac{N_t}{N_0}\right) \tag{1}$$

where LR = log reduction of bacteria count at time t; N_0 = initial bacterial concentration at time 0; N_t = final bacterial concentrations after a treatment time t.

The disinfection kinetic parameters of gram-negative bacteria at higher chlorine concentrations were determined by plotting inactivation data into Chick–Watson's empirical model [24–26]. Log reduction vs. time plots of survivors, helped to determine inactivation kinetics of pathogens [13,14]. The empirical logarithmic equation was expressed as:

$$\mathrm{Ln}\left(\frac{N_t}{N_0}\right) = -kCnT \tag{2}$$

where N = bacterial concentration at time t; N_0 = initial bacterial concentration at time 0; C = free chlorine concentration (mg/L); T = contact time (min); k = inactivation rate constant; n = co-efficient of dilution.

The objectives of this experimental research are as follows: (1) to assess the chlorine inactivation of six different gram-negative strains isolated from water supply points, (2) to assess the response of the bacterial strains, isolated from the chlorinated drinking water, (3) investigate the inactivation kinetics of bacterial isolates at varying contact times and chlorine concentrations using Chick–Watson model, (4) to find out the decay rate, CT factor, 2 and 3 log reduction of each isolated strain (5) to finally, analyze the statistical significance of the results obtained (using a statistics calculator SPSS 25).

2. Methodology

2.1. Isolates and growth conditions

Six strains namely *Providencia rustigianii* (*P. rustigianii*), *Pseudomonas aeruginosa*, *Enterobacter*, *Proteus myxofaciens*, *Morganella morganii* subsp. *sibonii* and *Morganella morganii* subsp. *morganii* was repeatedly isolated from treated water. The strains were identified by microbiological techniques (Membrane Filtration Technique), further identified by gram staining and biochemical test strips (HiMedia Laboratories Pvt. Ltd., A-516, Swastik Disha Business Park, Via Vadhani Ind. Est., LBS Marg, Mumbai-400086, India, Enterobacteriaceae Identification Kit, containing 13 biochemical test media and 12 sugar fermentation media). These isolates were repeatedly obtained after the final disinfection of water using sodium hypochlorite, maintaining an RFC level of 0.5–1.0 mg/L. Interestingly, these strains were tested for their chlorine response *in-vitro*. All six strains were found to resist 0.5 to 1 mg/L of RFC at a contact time of 30 min [28,29].

2.2. Maintenance of pure culture

Subcultures for all the strains were maintained in plate count agar (PCA). Another set was maintained on saline (0.85% NaCl) at pH 7.0–7.2. The culture was serially diluted in saline to get a final concentration of cells ranging from 6.0×10^{-4} to 17.0×10^{-4} CFU/mL. This range of cells served as blank containing 4.79–5.23 $\log_{10}$ CFU/mL.

2.3. Survival assay in-vitro in presence of RFC

Each isolate were subjected to a varying set of RFC concentrations ranging 0.5, 1.5, 2.5 and 3.5 mg/L respectively. The strain *P. rustigianii* showed *in-vitro* resistance to 3.5 mg/L. Hence, it was subjected to further treatment using a high dose of RFC concentrations of 5 and 7 mg/L. Chlorine demand free water was used for making the test preparations. The test was carried out at 27°C and pH 7.2; 1 mL of sample was taken from 1st set after 15, 30, 60, 120 and 180 min and plated on PCA (HiMedia). The process

was repeated for the 2nd, 3rd and 4th sets. The plates were incubated at 37°C for 24 h. After the enumeration of the colonies, the counts were expressed as $\log_{10}$ CFU/mL. Reaction tubes were continuously mixed (250 rpm) by using an overhead stirring apparatus equipped with sterile stainless steel paddles. Chlorine concentrations were determined by the *N*,*N*-dimethyl-*p*-phenylenediamine colorimetric method. Samples were plated from the reaction tubes at desired exposure times, and the action of chlorine was immediately neutralized by the addition of 0.5 mL of 10% (wt./v) sodium thiosulphate. Reaction tube containing no chlorine served as controls for determining unexposed concentrations of the bacteria [13,14,30–34].

2.4. Statistical analysis

The significance of the test was calculated by finding the differences between the means at a p level of <0.05. For the first-order kinetics of inactivation, $\log_{10}$ transformed data were used to determine the 2–3 log reduction and inactivation rate/decay rate (–*k*). The means for the inactivation of isolates at each exposure time were plotted in a semi-log graph. The first-order model was used to describe the inactivation rate using, Chick–Watson's rate kinetics model: $\ln N_t/N_0 = -kCnT$), where t = time in seconds, N_t = CFU/mL at any time t, N_0 = CFU/mL at time zero, Cn = concentration of RFC and $-k$ = the inactivation rate in min^{-1}. A regression analysis using least squares was conducted for experiments with each individual isolate and for the mean values for each type of isolates to determine the inactivation rates ("*k*" values) [26].

3. Results

In the present experimental investigation, the decay of free chlorine in the water was also studied as the chlorine decay helps to highlight the degradation rate of chlorine in the water. It helps to determine the RFC left in the water to inactive viable cells. In our observation it was found that 0.5 mg/L residual chlorine replenishes within 60 min of contact time, 1.5, 2.5 mg/L RFC reduces to half the concentration within 60 min of contact time, RFC 3.5 mg/L reduces to 2 mg/L after 60 min of contact time. After 180 min the residual effect of all the diluents 1.5, 2.5 and 3.5 replenishes absolutely out of the system. The plotted graphs (Figs. 1–6) show that inactivation kinetics, differ significantly for the six isolates (n = 6) at $p < 0.05$. At the recommended free residual chlorine of 0.5 mg/L chlorine tolerant isolate *P. rustigianii*, was reduced from 5.23 log to only 4.03 log after 30 min contact time. The graphs plotted (Figs. 7–12) using Chick–Watson's empirical formula shows that at a higher dose of chlorine and with the increased contact time, a marked 2 log inactivation ($T_{99\%}$) may be achieved. *In-vitro* inactivation kinetics study revealed a very high rate of bacterial kill overtime of *P. rustigianii*. Chlorine dose of 5 mg/L at 180 min and 7 mg/L at 120 min enabled 3 log inactivation ($T_{99.9\%}$), a relatively very high requirement of RFC to completely inactivate chlorine tolerant *P. rustigianii*. Other chlorine tolerant strains like *Proteus myxofaciens*, *Morganella morganii* subsp. *sibonii*, *Pseudomonas aeruginosa* and *Enterobacter* were poorly removed at 0.5 mg/L RFC for 30 min. 3 log inactivation ($T_{99.9\%}$) of these pathogens (n = 4) was achieved at 3.5 mg/L within 120 min. *Morganella morganii* subsp. *morganii*, was somewhat less tolerant to chlorine; 3 log inactivation was achieved with greater efficacy at 1.5 mg/L available free chlorine within 60 min.

The regression values (Table 1) ranged from $R^2 = 0.452$ to 0.880, significant differences at $P < 0.05$, were found in the inactivation rate constant of the tested isolates at $P < 0.05$. The standard operational guidelines demand the removal of pathogens from water treatment systems. The greater removal efficiency of isolates may be achieved at higher chlorine doses of 2.5–3.5 mg/L compared to Log inactivation at 0.5 mg/L in 30 min. Figs. 7–12 presented below, depicts a CT value of 3.5 mg/L at 60 min to produces greater than 4 log inactivation among the bacterial pathogens in this study and thus may be considered the optimal concentration required for deactivation of these strains. The presence of higher chlorine species reacting with bacteria cells causes greater inactivation of bacteria. Bacterial inactivation was rapid within the first 15 min while the inactivation rate slowly declined afterward with an increase in contact time. Findings reveal an overall 3.5 mg/L free chlorine may be considered an effective dosage for the

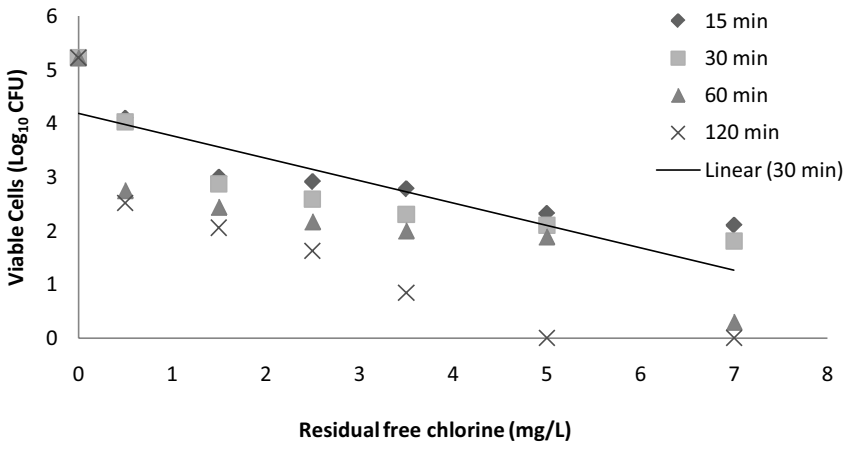

Fig. 1. Survival curves corresponding to the inactivation of *Providencia rustigianii* RFC at 27°C, pH 7.2.

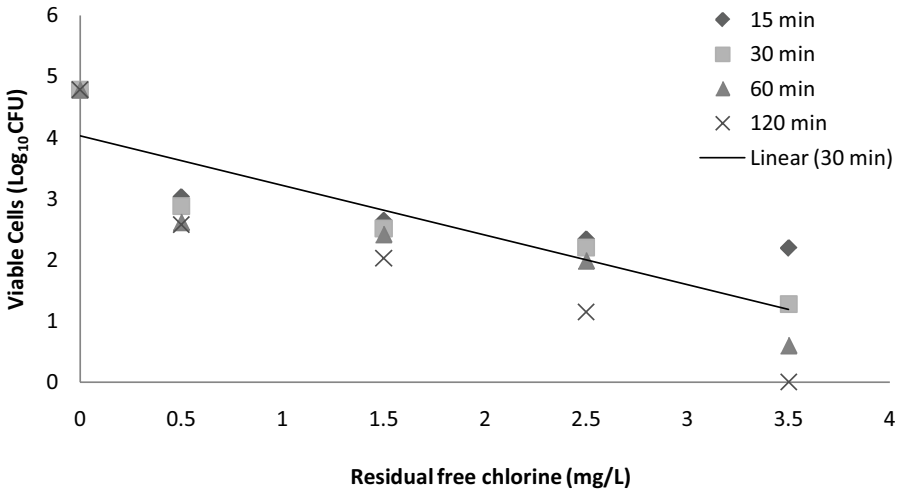

Fig. 2. Survival curves corresponding to the inactivation of *Proteus myxofaciens* RFC at 27°C, pH 7.2.

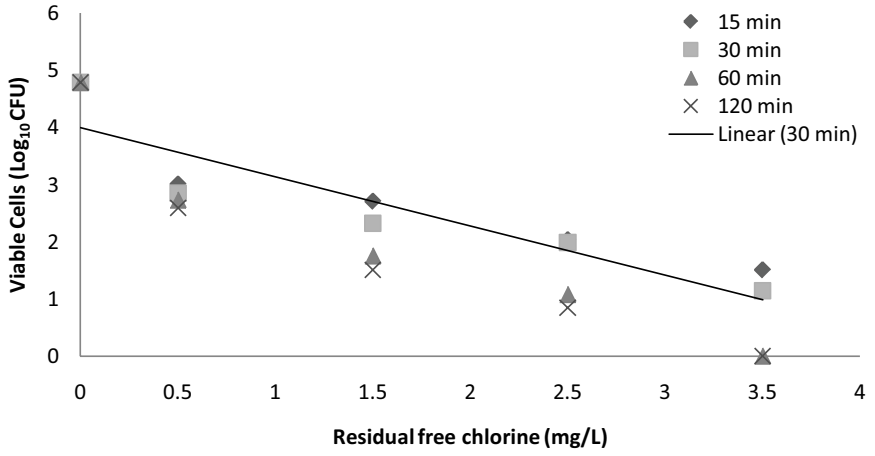

Fig. 3. Survival curves corresponding to the inactivation of *Pseudomonas aeruginosa* RFC at 27°C, pH 7.2.

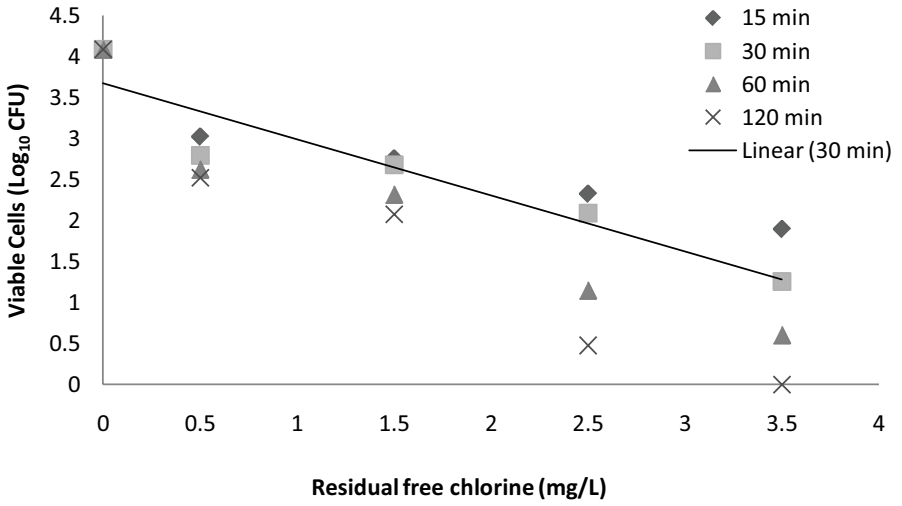

Fig. 4. Survival curves corresponding to the inactivation of *Enterobacter* RFC at 27°C, pH 7.2.

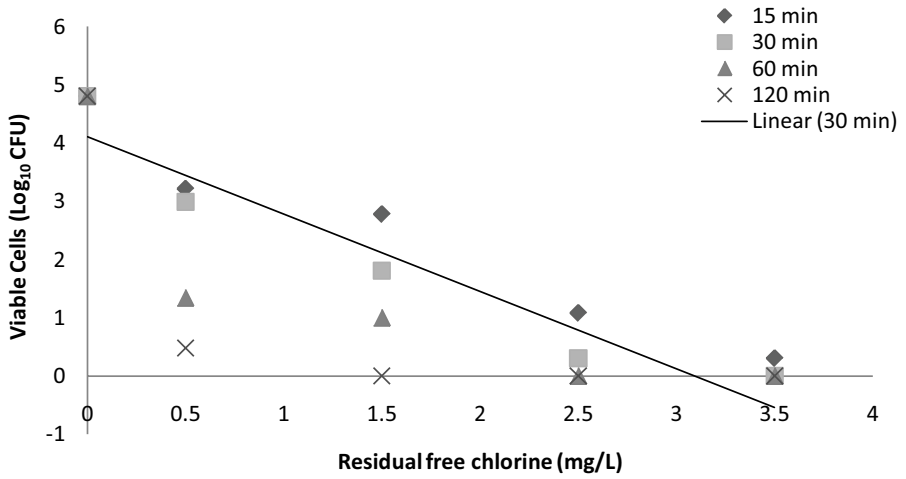

Fig. 5. Survival curves corresponding to the inactivation of *Morganella morganii* subsp. *morganii* RFC at 27°C, pH 7.2.

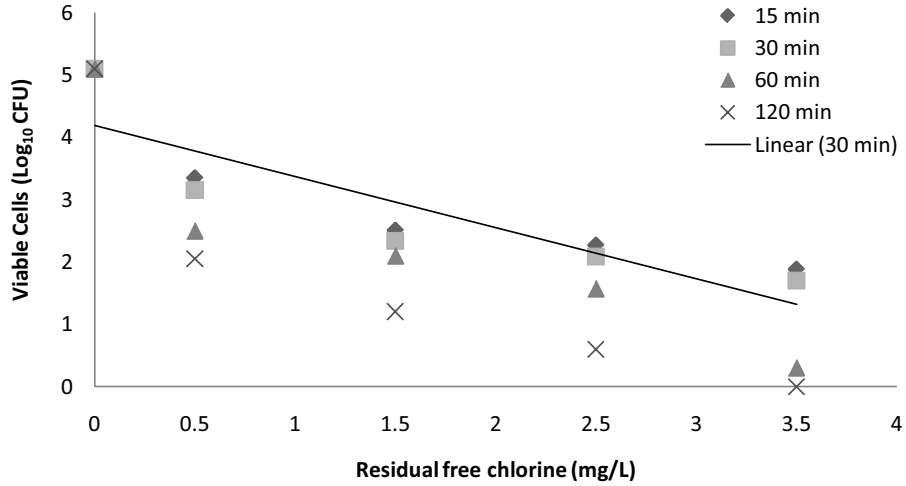

Fig. 6. Survival curves corresponding to the inactivation of *Morganella morganii* subsp. *sibonii* RFC at 27°C, pH 7.2.

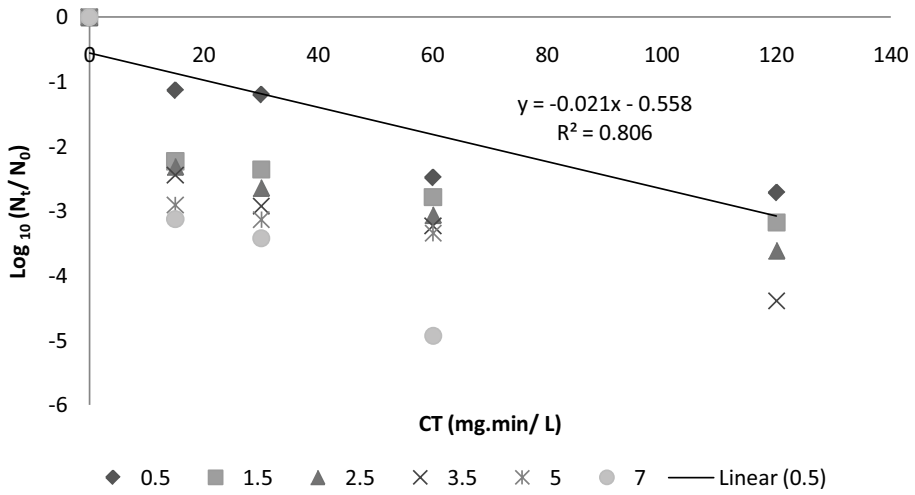

Fig. 7. The decay of *Providencia rustigianii* at varying residual free chlorine concentrations and varying contact time.

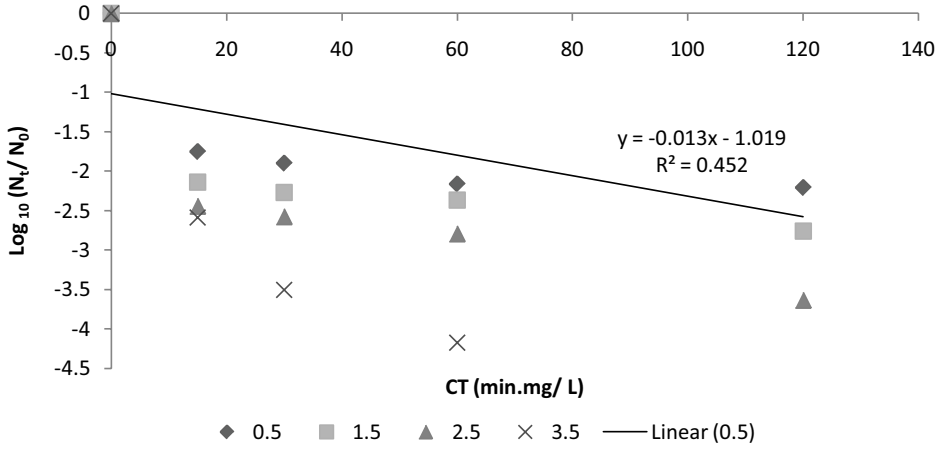

Fig. 8. The decay of *Proteus myxofaciens* at varying residual free chlorine concentrations and varying contact time.

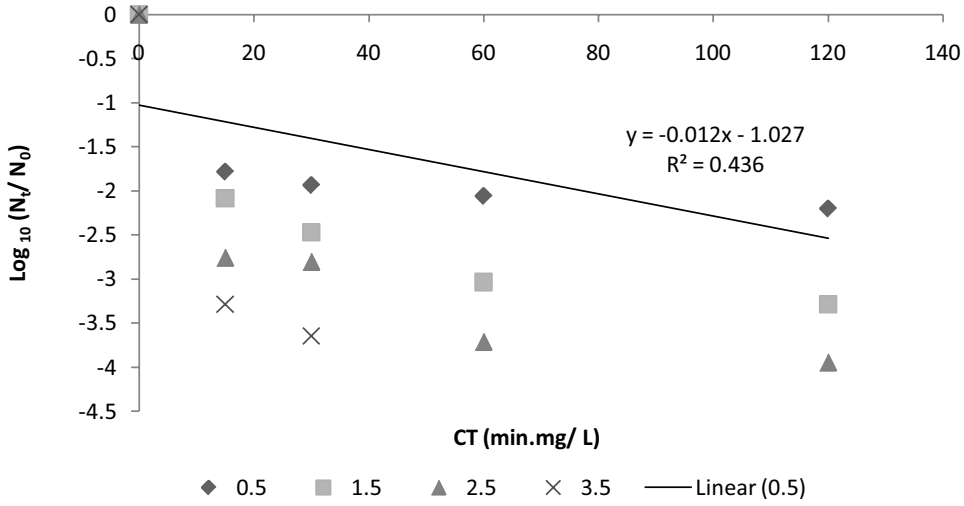

Fig. 9. The decay of *Pseudomonas aeruginosa* at varying residual free chlorine concentrations and varying contact time.

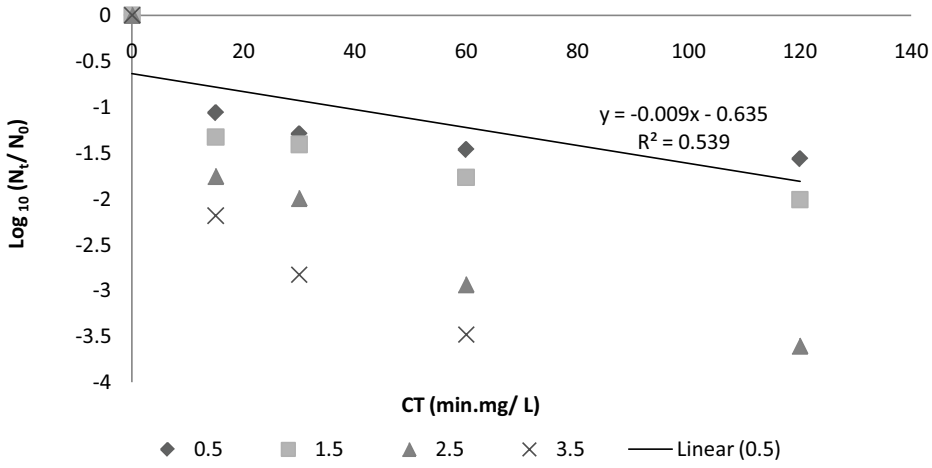

Fig. 10. The decay of *Enterobacter* at varying residual free chlorine concentrations and varying contact time.

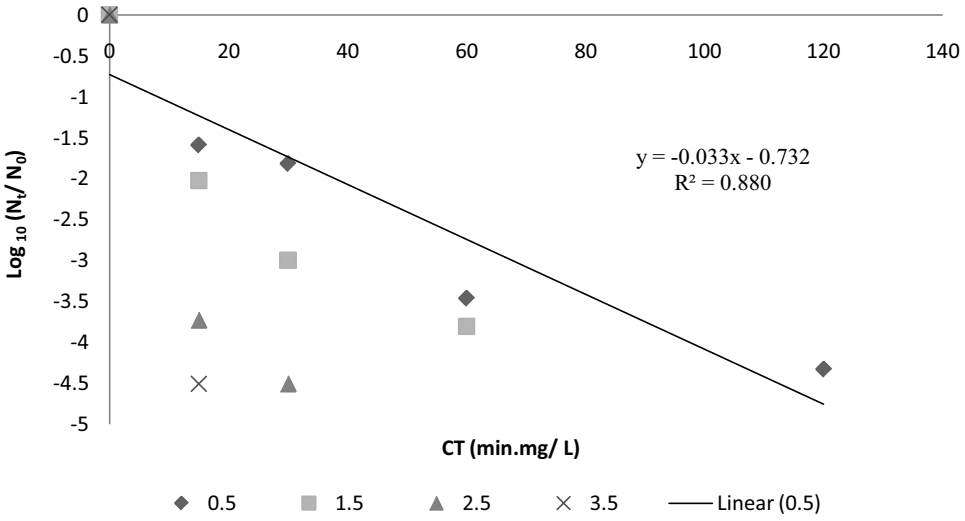

Fig. 11. The decay of *Morganella morganii* subsp. *morganii* at varying residual free chlorine concentrations and varying contact time.

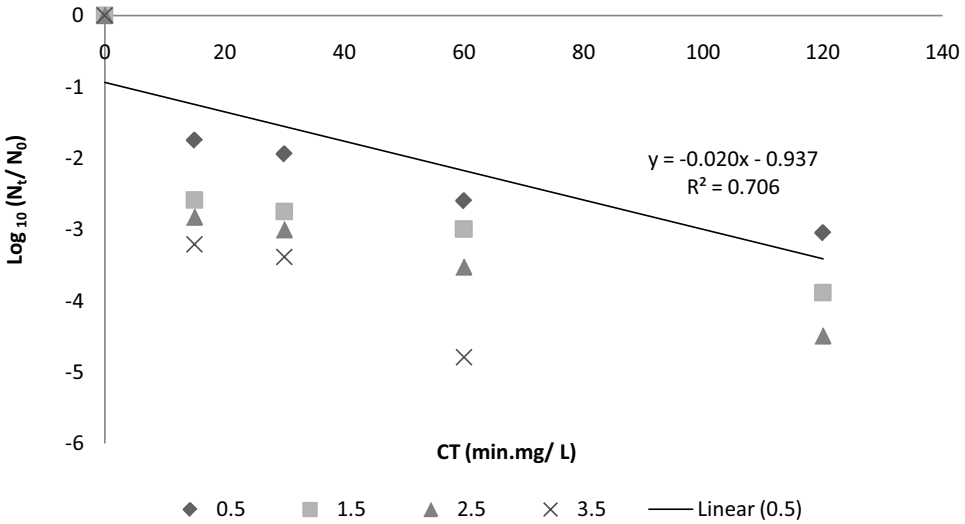

Fig. 12. The decay of *Morganella morganii* subsp. *sibonii* at varying residual free chlorine concentrations and varying contact time.

inactivation of 99.9% pathogens within 60 min and 99.99% effective within 120 min. A RFC dose so high may not be maintained within the water distribution lines but may be considered well enough as an effective dose for controlling chlorine tolerant bacteria. The guidelines for safe drinking water seldom allow such a high level of RFC, as it is detrimental to public health.

4. Conclusion

Chlorination is the most effective method for water disinfection that provides residual protection against bacteria, viruses and other pathogens in water. The residual effect of chlorine ensures protection against recontamination and ensures safety. Our experimental study using Chick–Watson model implies a detailed examination of bacterial inactivation using chlorine. Findings reveal that overall 3.5 mg/L free chlorine may be considered an effective dosage for the inactivation of 99.9% pathogens within 60 min and 99.99% effective within 120 min. Though chlorination is a widely effective practice in the water treatment system yet may harbor potentially chlorine resistant forms. Difficulty in chlorine disinfection may arise if any chlorine tolerant pathogenic form perpetually and persistently lasts longer inside a closed water distribution system. Ozonization may be a good alternative in the treatment of such a potentially resistant pathogen. Though effective it is a costly method with no residual effect to protect treated water from further recontamination. Over the decade chlorination has proven to adequately control pathogens when compared to other disinfection processes. The occurrence of pathogenic bacteria in otherwise high-quality drinking water with high chlorine content has recently shown that the maintenance of a chlorine residual cannot be relied on to prevent public health problems. The available reports and present experimental investigation

Table 1
Log inactivation of gram negative isolates at pH 7.2, temperature 27°C, RFC 0.5 mg/L, contact time 30 min. (Chick–Watson model: $\log N_t/N_0 = -kCnT$)

Isolates (n = 6)	Initial inoculum ($\log_{10}$ CFU)	RFC (mg/L)	Viable cells after 30 min exposure ($\log_{10}$ CFU/mL)	CT	Log inact. (%)	Decay rate, k (min^{-1})	R^2
Pseudomonas aeruginosa	4.79	0.5	2.85	15	>90	0.29	0.436
		1.5	2.32	45	>90	0.12	
		2.5	1.98	75	91.30	0.08	
		3.5	1.14	105	93.60	0.06	
Enterobacter	4.08	0.5	2.79	15	>90	0.17	0.539
		1.5	2.67	45	92.90	0.07	
		2.5	2.08	75	93.80	0.06	
		3.5	1.25	105	92.00	0.07	
Morganella morganii subsp. *sibonii*	5.09	0.5	3.15	15	>90	0.29	0.706
		1.5	2.34	45	91.07	0.08	
		2.5	2.08	75	90.80	0.09	
		3.5	1.70	105	92.58	0.07	
Morganella morganii subsp. *morganii*	4.80	0.5	2.98	15	>90	0.27	0.880
		1.5	1.80	45	>90	0.15	
		2.5	0.30	75	>90	0.13	
		3.5	0.00	105	90.9	0.10	
Proteus myxofaciens	4.80	0.5	2.87	15	>90	0.29	0.452
		1.5	2.51	45	>90	0.11	
		2.5	2.20	75	92.10	0.07	
		3.5	1.27	105	92.10	0.07	
Providencia rustigianii	5.23	0.5	4.03	15	>90	0.18	0.452
		1.5	2.87	45	>90	0.12	
		2.5	2.59	75	91.91	0.08	
		3.5	2.30	105	93.80	0.06	

suggest different sensitivity of bacterial strains towards chlorine. The results of the present study indicate high tolerance of gram-negative strains (n = 6) towards chlorination. Evaluation of other gram-negative isolates under differing environmental conditions would be commendable for progressive concern. A detailed ongoing experimental investigation on the decay of free chlorine in water is under study. The study would help to determine the RFC left in the water to inactive viable pathogens.

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