

Effect of bacterial growth stage on resistance to chlorine disinfection

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ABSTRACT

The mechanisms and factors that affect microbial resistance to chlorine disinfection have not been fully elucidated. In this study, we investigated the impact of the cell growth stage on chlorine disinfection efficiency. Specifically, we evaluated the impact of the growth stage on chlorination resistance by comparing the inactivation efficiencies of two indicator bacterial strains (*Escherichia coli* K12 and *Escherichia coli* O157:H7) obtained from various growth phases, using Chick-Watson kinetic parameters. For both *E. coli* strains (K12 and O157:H7), the inactivation rate constants are the lowest at stationary phase (0.19 and 0.32) compared to those at initial lag (0.54 and 0.76) and exponential growth phase (0.63 and 0.69), respectively. These results suggested that the abundance of resistant subpopulations increases at stressed stationary conditions and *E. coli* cells obtained from the stationary growth phase exhibited more resistance and lower inactivation efficiency compared to those from the lag and exponential phases. This implies that microbes in wastewater treatment process with varying solids retention times (SRTs, which indicate growth rates) may show different extents of chlorine resistance. Comparison of the coefficient of dilution (n) values in both *E. coli* strains for the various growth phases suggest that cells seem to be more sensitive to disinfectant concentration at the stationary-lag phase than that at the exponential stage. Comparing the two *E. coli* strains, higher inactivation rates were observed for the pathogenic O157:H7 than for K12 at different stages of growth. The strain-to-strain variability in survivability to chlorine exposure has to be considered when selecting indicator microorganisms for water quality monitoring.

Key words | chlorine disinfection, *E. coli*, growth stage, inactivation model, indicator organism, resistance

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INTRODUCTION

Chlorination of water and wastewater is the primary recognized measure to ensure low levels of pathogens in potable and recreational waters (Rice *et al.* 1993). However, microbial resistance to chlorination has been recently observed in both lab studies and in full-scale chlorine disinfection practice for water and wastewater treatment (Hoefel *et al.* 2005). Resistance has been defined as the temporary or permanent ability of bacteria to remain viable and/or multiply under conditions that would destroy or inhibit other members of the strain (Cloete 2003). So far, different factors have been identified for the chlorination disinfection inefficiency observed due to resistance phenomena. Inadequate residual levels of chlorine due to the interference of chlorinated organic amino-nitrogen compounds (Scully *et al.* 1999), the shielding effect provided by the background

matrix (Hoff 1978), the physical-chemical properties of the water and various operating conditions (Ridgway & Olson 1982) have been recognized to contribute to the observed resistance.

In addition to operational and environmental factors, physiological features, adaptation or genetic changes of different bacterial strains can play a role in the diverse resistance to chlorination. In particular, previous studies (Wojcicka *et al.* 2007) have found that indigenous bacteria isolated from different environments or subjected to stress conditions (i.e. starvation) (Lisle *et al.* 1998) have developed resistance or resistant phenotypes to various disinfection methods. Bacterial resistance to chlorine was also found to follow a biphasic process (Lisle *et al.* 1998), as for the majority of antimicrobial compounds (Keren *et al.* 2004).

Furthermore, the synthesis of unique proteins in response to stress has been widely documented (Blom *et al.* 1992) and *E. coli* exposed to chlorine seem to induce a specific set of proteins, some overlapping with heat shock and carbon starvation, which make them less susceptible to the disinfectant action. Several studies have also assessed the potential role of bacterial quorum sensing and the consequent regulation of cellular functions based on the surrounding cell density in chlorination efficiency (Lazazzera 2000).

The existence of persister cells within a bacterial population (which are typically 10^{-6} – 10^{-4}) have been recently observed after antibiotic treatments (Keren *et al.* 2004) and, therefore, might also refer to other antimicrobial processes. Persisters are surviving cells that neither grow nor die in presence of biocidals and they might be responsible for the high levels of tolerance to antimicrobials in biofilms. The mechanisms involved in these processes of persister formation are quite complex and they are still not fully understood. Therefore, further investigation is needed on microorganisms' resistance to chlorine disinfection, especially considering that chlorine is still the most applied disinfection method worldwide. It is known that microorganisms have different morphological and physiological features at different growth stages (Regine 2000) and, for some microorganisms, the susceptibility to antibacterial agents decreases with increasing growth rate (Mah & O'Toole 2001).

Studies of bacterial resistance to antibiotics have shown that the persistency phenomena of bacteria are strongly related to the bacterial growth stage. For example, it was found that persisters are not produced at the early exponential stage in the cell cycle, but only in the stationary phase (Keren *et al.* 2004). Higher antibiotic resistance during the stationary phase were associated with the induction of stress resistant genes such as σ^S factor (Dukan *et al.* 1996), while for the exponential stage, other σ^S -independent resistance mechanisms, such as the induction of the OxyR regulon in the adaptive response to H_2O_2 , were observed. In light of the varying persistence during different growth stages, we hypothesize that the cell growth stage will impact on the chlorine disinfection efficiency during water treatment and consequently the overall design of disinfection systems.

In this study, we investigated the effect of growth stages on chlorine inactivation efficiency and associated resistance for two *E. coli* strains, namely K12 MG1655 (laboratory model organism) and O157:H7 (enterohemorrhagic-pathogen strain). Chlorine inactivation rates and kinetic constants based on the traditional Chick-Watson model

were determined and compared for the two *E. coli* strains at various growth stages. The varying sensitivity and resistance to chlorine disinfection between the two *E. coli* strains at different growth phases is revealed and the implications for water and wastewater disinfection treatment processes are discussed.

MATERIALS AND METHODS

Culture sources and growth

Bench-scale inactivation studies were carried out with reference strains of *E. coli* K12 and *E. coli* O157:H7. *E. coli* is often used as a model organism for disinfection studies since it is known to be very sensitive to free chlorine at doses consistent with those in water distribution systems (Helbling & VanBriesen 2007). *E. coli* K12 MG1655 was chosen as a laboratory model bacterium, while *E. coli* O157:H7 is an enterohemorrhagic strain often implicated in foodborne and waterborne outbreaks worldwide (Meng *et al.* 1998). The two *Escherichia coli* strains were kindly provided by Dr. Kim Lewis in the Biology Department at Northeastern University (MA, U.S.). The bacterial cultures were grown overnight in fresh Luria-Bertani media for the chlorine disinfection inactivation experiments.

Growth phase and sampling time

Overnight bacterial suspensions of stationary *E. coli* were diluted in a 2L Erlenmeyer flask containing fresh LB media to achieve an initial cell concentration of 10^5 cfu/100 ml and then incubated at 37 °C for growth monitoring. Aliquots of samples were removed at different time points reflecting different bacterial growth stages, which were predetermined with a growth curve (Figure 1): 1.5 h: lag phase, 2.5 h: early exponential phase, 5.5 h: mid-exponential phase, 7.5 h: late exponential phase, 10–12 h: stationary phase. No differences in lag, exponential and stationary phase length durations were found among the K12 and O157:H7 *E. coli* strains.

Chlorine disinfection inactivation tests

Samples were washed twice in a phosphate buffer solution (PBS: 42.5 mg/L KH_2PO_4 , 405.5 mg/L $MgCl_2 \cdot 6H_2O$) before subject to the disinfection test. Tests were performed at 20 °C in Erlenmeyer flasks containing 200 ml of PBS inoculated with the pre-washed bacteria. A stock solution of sodium hypochlorite (250 mg/L) was prepared by diluting

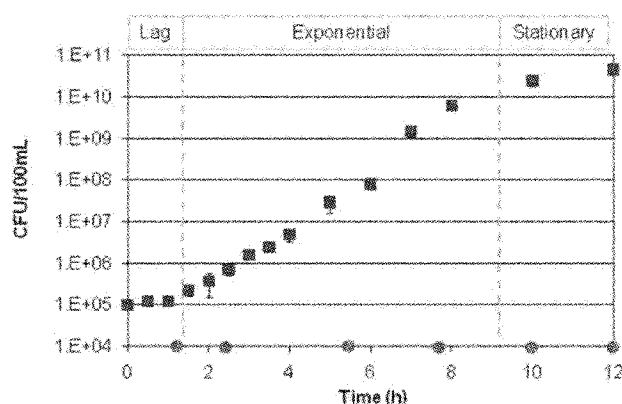


Figure 1 | *E. coli* K12 growth curve (■) with distinct lag, exponential and stationary phases and sampling time points (●) chosen for testing.

the commercial Clorox in 1L of deionized water and dosed accordingly to obtain a series of free residual chlorine concentrations in the range of 0.1 to 8 MgCl₂/L. One treatment per each concentration was performed and triplicate samples were taken for analysis. The residual chlorine concentrations was determined by the *N,N*-diethyl-*p*-phenylenediamine (DPD) method (APHA, WPCF 1992). Contact time between bacteria and the disinfectant was fixed at 10 min and the reaction was instantaneously stopped by the addition of excess 10% sodium thiosulfate solution to quench the disinfectant action. Enumeration of bacteria was performed via membrane filtration (APHA, WPCF 1992) in triplicate and filters were laid onto LB plates and cultured at 37 °C overnight for counting.

Chlorine inactivation modeling and determination of kinetic parameters

Inactivation data obtained were fitted to the empirical Chick-Watson model for disinfection kinetic parameters determination and inactivation rates comparison. The Chick-Watson model is a traditional model for predicting bacterial inactivation by chlorine disinfection, and it is often applied for disinfection process design. The reaction is described by the following equation:

$$\log \frac{N}{N_0} = -kC^nT \quad (1)$$

N , N_0 = bacterial concentration at contact time t and time 0 (cfu/100 ml), C = residual chlorine concentration (MgCl₂/L), T = contact time between bacteria and disinfectant (min), k = rate constant for a specific microorganism and set of conditions, n = coefficient of dilution.

The values of the first order disinfectant decay rate (k) and dilution coefficients (n) for each experiments were determined by least-squares regression analysis in which the sum of the squares of the difference between observed and predicted log-survival ratios was minimized by finding the optimal values of the kinetic parameters. The computations were conducted using Matlab v. 2009. A statistical p -value from paired t-test was used for data sets comparison.

RESULTS AND DISCUSSION

Effect of growth stage on chlorine disinfection survival ratio and inactivation rates

Results from inactivation tests showed differences in survival ratios of *E. coli* K12 and O157:H7 at different stages of growth after 10 min exposure to 1 mg/L of chlorine (Figure 2). Both *E. coli* strains exhibited higher survival ratios [$-\log(N/N_0)$] in the lag phase than the ones obtained during the following exponential and stationary stages of growth. For instance the model strain *E. coli* K12 was significantly less susceptible to inactivation during the stationary phase (lower survival ratio, 2.12) than in the exponential (4.59) and lag stage (4.96). A relatively higher susceptibility to chlorine for the *E. coli* O157:H7 strain than the *E. coli* K12 was observed for all growth phases, as indicated by the lower inactivation rates by 41% (lag), 40% (exponential) and 91% (stationary) for the different growth stages. This indicates that the pathogenic strain has a higher sensitivity to chlorine than the lab strain under the same experimental conditions.

Evidence of the impact of the growth stage on cell behavior to disinfectant has been reported previously with

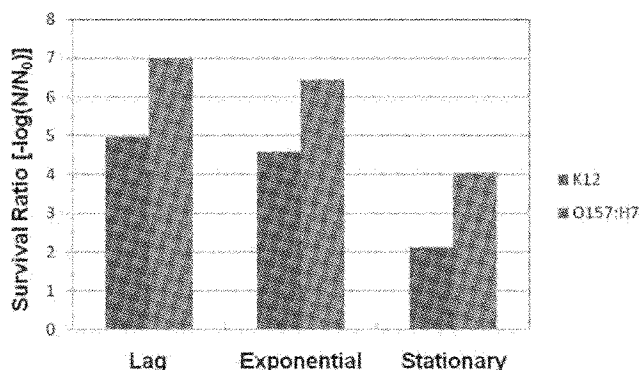


Figure 2 | Survival ratio at different growth stages of *E. coli* K12 and *E. coli* O157:H7 exposed to 1 mg/L of chlorine for 10 min.

divergent conclusions. Stewart & Olson (1992) reported that the growth stage did not impact resistance of *Klebsiella pneumoniae* to chloramines, while Carson *et al.* (1972) found that the survival of *Pseudomonas aeruginosa* exposed to acetic acid was halved (comparing the logarithmic exponential phase to the stationary phase cells). Therefore, it is clear that the microorganisms' genus or specie as well as the disinfectant type dictate if the growth stage plays a role in the persistence of cells after disinfection processes. A recent study by Kaymak & Haas (2008) also emphasizes the role of growth rate in bacteria inactivation showing that the degree of survival of stationary-phase *E. coli* (ATCC 13706) exposed to monochloramine is cell density-dependant. Bacterial growth stage may have direct implications on the efficiency of disinfection systems for water and wastewater treatments since the microbial growth rate is controlled by the operating system parameters (e.g. solids retention time). Therefore, plant-to-plant variability in the level of bacterial resistance to different disinfection methods is expected due to differences in bacterial communities and system operations.

Variation of chlorine disinfection kinetics at different growth stages

To further evaluate the impact of growth stage on the susceptibility of *E. coli* cells to chlorination, we applied a conventional chlorine inactivation model, Chick-Watson's law (Equation (1)), to determine and compare the inactivation kinetic parameters for cells from different growth stages. The inactivation effect of chlorine on

E. coli was described and compared using k and n , Chick-Watson model parameters, which quantify the survival rate in laboratory inactivation experiments.

The inactivation constant k is microorganism-specific and it also depends on experimental conditions. The microbes become non-viable at a rate governed by k , thus higher k values reflect faster bacterial inactivation. On the other hand, the average empirical coefficient n , so called coefficient of dilution, is a constant that represents the average number of disinfectant molecules in contact with an organism necessary to cause inactivation and encloses the relative weight of impact of the disinfectant concentration versus exposure time. In similar scenarios as the one reported in this study, for n greater than 1 the applied chlorine concentration has higher influence than contact time in bacterial inactivation. In many cases, the n value for the Chick-Watson law is found to be close to 1.0 and, therefore, when a fixed value of the product between concentration dosed and contact time (CT product) is considered, the degree of cell inactivation is also fixed (AWWA 1999).

In this study, the kinetic parameters determined based on the Chick-Watson model for chlorine inactivation were significantly different (paired p -value was 0.049 for k and 0.02 for n calculation) for the same cell strain from different growth stages (Table 1). For both *E. coli* strains, the inactivation rate constants k were the lowest at the stationary phase compared to those at the early lagging phase after cell transfer and at the following exponential phase. The inactivation constant k for *E. coli* K12 decreased from 0.63 at the exponential phase to 0.19 at the stationary phase. There seem to be a lag phase before the exponential phase, likely

Table 1 | Kinetic parameters of inactivation for *E. coli* K12 and *E. coli* O157:H7 by chlorine at different bacterial growth stages

	Growth phase	k	n	Inactivation equation
<i>E. coli</i> K12	Lag	0.54 (0.3642, 0.7208)	1.16 (0.4309, 1.896)	$\text{Log}(N/N_0) = -0.54C^{1.16}T$
	Exponential	0.63 (0.4703, 0.7926)	0.67 (0.2113, 1.119)	$\text{Log}(N/N_0) = -0.63C^{0.67}T$
	Stationary	0.19 (0.08777, 0.2943)	0.73 (0.4168, 1.048)	$\text{Log}(N/N_0) = -0.19C^{0.73}T$
<i>E. coli</i> O157:H7	Lag	0.76 (0.5696, 0.9571)	0.83 (0.3669, 1.285)	$\text{Log}(N/N_0) = -0.76C^{0.83}T$
	Exponential	0.69 (0.6441, 0.7386)	0.35 (0.2229, 0.4816)	$\text{log}(N/N_0) = -0.69C^{0.35}T$
	Stationary	0.32 (0.1922, 0.4553)	0.57 (0.3208, 0.8269)	$\text{log}(N/N_0) = -0.32C^{0.57}T$

Values between parentheses refer to 95% confidence interval.

due to the culture wash and transfer procedures when allocating the cells from the previous stationary growth condition into fresh media. A similar pattern was also observed with the *E. coli* O157:H7 strain, which exhibited a decrease in the inactivation rate constant through the different cell growth phases, with lag phase k value declined from 0.76 to 0.69 and 0.32 in exponential and stationary phases respectively. The decrease in k values indicates the general increase in resistance for *E. coli* cells at elder growth stages, likely due to the increase in the number of more resistant sub-populations as discussed in the introduction. The increased resistance to chlorination at a later stage of growth in bacteria may be promoted by various factors such as quorum sensing signaling mechanisms, initial cell density and general physiological and morphological modifications (Kaymak & Haas 2008).

By fitting our inactivation data to the Chick-Watson model, we determined n values from the two *E. coli* strains from various growth stages. So, from these data, the Chick-Watson model generates different equations at each stage of growth, which are reported in Table 1.

The coefficient of dilution determined ranged from 0.67 (exponential phase) to 1.16 (lag phase) for *E. coli* K12, and was in the range of 0.83 (lag phase) to 0.35 (exponential phase) for *E. coli* O157:H7. The value of the coefficient n seems to be noticeably lower at the exponential phase than those from lag and stationary stage for both *E. coli* strains tested. This indicates that the cells seem to be more sensitive to disinfectant concentration at stationary-lag phase than that at exponential stage. The significance of n in disinfection reactions is still controversial: it is believed that the dilution coefficient reflects the reaction order of the disinfection experiment, with the hypothesis that the death rate and the chemical reaction rate are identical. Therefore, a low Chick-Watson slope parameter may indicate an organism possessing a higher degree of innate resistance to the chemical or physical damage caused by the antimicrobial. This is in agreement with the results of our study. On the other hand, other reports (Lambert 2001) stated that the same interpretation of the slope parameter and the dilution coefficient as a reaction order is erroneous. Nevertheless, most studies (Berry *et al.* 2009) agree in assessing that the variation in n may be related to a combination of factors, including organism-specific physiology, inactivation conditions, pH, etc., so different n value will be generated in specific experimental conditions. These results clearly demonstrated that chlorine inactivation efficiency depends on the growth stage of cells and the abundance of resistant subpopulations increases at more stressed

stationary conditions. Results summarized in a previous report (Scully *et al.* 1999) also show the same resistant behavior to chlorine for other microorganisms such as *K. pneumoniae* cells and *Flavobacterium* species exposed to low nutrient (stress) conditions.

Comparison of varying resistance among different bacterial strains

Genetic differences among bacterial strains may lead to differences in the adaptive capabilities of the *E. coli* species to various environmental conditions or stresses. Although the two strains, the O157:H7 and the K12 strain, share an average nucleotide identity of 98.5%, they exhibit differences in their genomes and phenotypes. The possible role of genetically-based increase in resistance to disinfectants by natural selection has been speculated in previous studies, where chlorine-induced mutations were considered responsible for the enhanced sensitivity to the disinfectant by the progeny of chlorine-exposed cells (Hass & Morrison 1981).

In this study, differences in inactivation behaviors by chlorine between the non-pathogenic and the pathogenic *E. coli* were observed with the same experimental conditions (Table 1). In the comparison between the inactivation kinetics for the two *E. coli* strains tested, slightly higher inactivation rates were observed for the pathogenic O157:H7 than for K12 at different stages of growth (10% higher during exponential phase and 70% higher during the stationary growth phase). In addition, the results also show that for the K12 strain, the concentration of disinfectant seems to have a greater influence than contact time in the inactivation (higher n) than for the O157:H7. The n value estimated for the K12 strain was higher by 41% for lag phase, 89% for exponential and 28% for stationary phase than those for the *E. coli* O157:H7, respectively. These results suggest that *E. coli* O157:H7 is more sensitive to chlorine than *E. coli* K12, although the detailed mechanism responsible for this difference remains to be elucidated. Higher susceptibility to disinfectant or antimicrobial treatment of a wild strain than that for a lab strain has been reported before (Wojcicka *et al.* 2007). For example, it was found that environmental isolates of heterotrophic bacteria were generally more easily inactivated than the reference strains (Wojcicka *et al.* 2007) under the same experimental conditions.

Our results clearly demonstrated that there are significant variations in the susceptibility to chlorine disinfection among different *E. coli* strains, and in addition, the inactivation rates also differ for the same strain under various

growth stages. In water and wastewater treatment processes, different operating conditions may dictate the growth rate and stage of microorganisms including those indicators such as *E. coli*. Among the different operational parameters considered in the literature, the solids retention time (SRT) has been typically used in process design and control and it is of importance since it represents the sludge age, the average time the biomass stays in the treatment systems. For example, processing with a longer SRT leads to slower growth rates (e.g. near stationary condition) of microorganisms than those with a shorter SRT. And systems that have a fixed-film process have a longer SRT than those with a suspended activated sludge process. This implies that for disinfection process design and, for understanding the potential variability in the disinfection efficiency observed among different facilities, the upper-stream water and wastewater treatment processes that affect the microbial growth stages need to be taken into consideration. Furthermore, when selecting indicator microorganisms to detect fecal contamination for water quality monitoring, the varying resistance to disinfectant among different strains has to be considered.

CONCLUSIONS

The objective of this work was to quantitatively assess the impact of the cell growth stage on the susceptibility of non-pathogenic (K12) and pathogenic (O157:H7) *E. coli* to chlorine disinfection. The results led to the following conclusions:

1. For both *E. coli* strains (K12 and O157:H7) evaluated, the bacterial cells from different growth stages exhibited varying susceptibility and resistance to chlorination, as indicated by the statistically different inactivation kinetic parameters determined for cells from different stages of growth.
2. Both evaluated *E. coli* strains exhibited higher resistance to chlorination at the stationary growth stage than those from lag and exponential phases. Cells from the exponential growth stage, however, seem to be less sensitive to the disinfectant concentration than those from other growth stages as indicated by the coefficients of dilution values comparison.
3. There was strain-to-strain variation in terms of their inactivation behaviors towards chlorination and the pathogenic strain O157:H7 seemed to be more sensitive and more susceptible to chlorine than the lab-strain K12.
4. Chlorine inactivation kinetics of bacterial populations grown in water and wastewater treatment systems with different operation conditions (e.g. SRTs) are expected to be different due to growth-stage associated resistance phenomena, therefore modification of plant design and/or operating conditions may be necessary.

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