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Development of Resistance in *Escherichia coli* Against Repeated Water Disinfection

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Pathogen resistance against common disinfectants in drinking water treatment could have serious public health implications, particularly in potable water reuse. Frequent disinfection in potable water reuse has a potential to facilitate resistance development. This study investigated resistance development in *Escherichia coli* against repeated monochloramine and ferrate disinfection. *E. coli* cultures repeatedly treated with monochloramine developed resistance after 12 + treatment rounds, whereas repeated ferrate disinfection did not lead to resistance development. Monochloramine induced cells into the viable but nonculturable (VBNC) state in initial disinfection rounds; however, repeated monochloramine treatment caused increases in culturability, which corresponded to decreases in the fraction of VBNC cells post-disinfection. The cumulative number of disinfection episodes (~12 times) rather than treatment frequency (e.g., every 48, 96, or 144 h) played a critical role in resistance development against monochloramine. In addition to preventing resistance, ferrate effectively inactivated (>3-$\log_{10}$) the evolved monochloramine-stressed cultures, suggesting that the evolutionary adaptations against monochloramine were ineffective against ferrate. The lack of resistance against ferrate renders it a promising disinfection agent that deserves further assessment. This work's findings demonstrate that repeated disinfection coupled with the type of applied disinfectant can influence bacterial resistance development. Therefore, current and future water disinfection schemes, especially within potable water reuse, need regular monitoring to evaluate the resistance profile of pathogenic bacteria.

Keywords: monochloramine, ferrate (VI), VBNC (viable but nonculturable), cAMP, culturability, water reuse

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INTRODUCTION

Advances in the drinking water treatment industry significantly reduced mortality and illnesses caused by waterborne pathogens. Specifically, disinfection has led to enhanced pathogen removal, thereby limiting the spread of waterborne diseases. However, drinking water-associated outbreaks continue to reoccur in the United States (Gerberding et al., 2008; Benedict et al., 2017). Most recent surveillance data of waterborne illnesses published by the Centers for Disease Control and Prevention (CDC) reported 66 outbreaks associated with drinking water during 2019–2020, which accounted for at least 337 illness cases (CDC, 2020). According to CDC, 45 and 30% of the outbreaks and illnesses were triggered by exposures to *Legionella* and Norovirus infections, respectively (CDC, 2020).

The growing global water shortages further exacerbate challenges related to drinking water treatment and disinfection. For example, although the reuse of treated wastewater effluent could offer an alternative and sustainable potable water source, it is challenged by the public's perception of potential health risks (Pecson et al., 2017; Soller et al., 2018; Kantor et al., 2019). The application of advanced water treatment (AWT), consisting of a multi-barrier treatment system, helps minimize potential health risks and improves public acceptance (USEPA, 2018). However, despite AWT, evidence of microbial growth in water distribution systems or reservoirs has been found (Park and Hu, 2010; Kantor et al., 2019). Reduction in microbial susceptibility to disinfection could have drastic implications for direct and indirect potable water reuse (i.e., DPR and IPR). As a result, an improved understanding of factors that impact microbial susceptibility to disinfectants is critical for predicting the effectiveness of disinfection, especially for potable water reuse. Various source water physical, chemical and microbiological characteristics, such as temperature, pH, organic content or microbial load, are known to interfere with disinfection effectiveness (Winward et al., 2008; Amiri et al., 2010). However, it is unknown if conditions relevant to potable water reuse, e.g., frequent disinfection, could facilitate bacterial resistance against disinfectants. A key question is whether surviving fractions of bacterial populations with altered physiology following disinfection go on to regrow, and after frequent disinfection, ultimately, develop resistance to disinfection. Hence, there is a need to determine the effect of frequent disinfection on bacterial resistance development for improved public health assessments of potable water reuse (USEPA, 2018).

While a significant body of literature has investigated the development of bacterial resistance to antibiotics, the influence of frequent disinfection on resistance development in bacteria is not well understood (van den Bergh et al., 2016; Levin-Reisman et al., 2017). Various defense responses are triggered against chemicals in bacterial cells such as cell damage repair, neutralization of toxic constituents, restoration of cellular homeostasis, or target inactivity through reduced cell growth and metabolism (Dukan et al., 1996; Carmel-Harel and Storz, 2000; Wang et al., 2009; Zhao et al., 2016; Ayrapetyan et al., 2018; Zhang et al., 2018). Collectively, defense mechanisms alter bacterial physiological responses at the phenotypic and, often, the genetic level, which decreases the susceptibility of subsequent generations to chemical exposure. However, reports of mechanisms contributing to bacterial resistance/tolerance against disinfectants in the literature are scarce (Farkas-Himsley, 1964; Haas and Morrison, 1981; Leyval et al., 1984; Hoff and Akin, 1986; Inatsu et al., 2010; Gundlach and Winter 2014). Additionally, the majority of studies have focused on resistance development against chlorine-based disinfectants due to their widespread use, though the studies show contradictory results. Farkas-Himsley (1964) found deliberate exposure of *Escherichia coli* to high chlorine concentrations in buffered solutions resulted in chlorine-resistant strains. Gundlach and Winter (2014) noted an incremental increase in resistance to hypochlorous acid (3.5–9.2 mM) in *E. coli* following multiple exposure cycles to the disinfectant in rich growth media. The buildup of resistance was found to be driven by the constitutive expression of OxyR-regulated genes, which enhanced the cells' oxidative stress response (Gundlach and Winter 2014). Meanwhile, other studies have shown that high resistance to hypochlorous acid (3–7 mM) in *E. coli* occurred as a result of decreased cyclic adenosine monophosphate (cAMP) levels and cAMP receptor protein (CRP) and, in turn, the accumulation of cellular general stress response regulator (RpoS) (Barth et al., 2009).

In contrast, Haas and Morrison (1981) showed that repeated chlorination of *E. coli* did not yield highly tolerant strains. However, in this study bacterial sensitivity may have been altered by the protocol's intermediate sub-culturing step in rich growth media between each chlorine exposure (Haas and Morrison, 1981). Similarly, Inatsu et al. (2010) showed no change in resistance against sodium hypochlorite among *E. coli* cultures repeatedly treated with the disinfectant. Fewer studies investigated resistance development in response to other disinfectants. A study using fast-acting hydroxyl radicals as the active disinfectant showed no resistance development in *E. coli* following 40 exposure cycles in phosphate-buffered saline (Ikai et al., 2013). As such, differences in disinfectants' action and cultivation protocols relevant to source water chemistry could influence mechanisms that lead to bacterial resistance/tolerance development.

In this work, the development of resistance in the model bacterium, *E. coli*, due to frequent (every 48 h) exposure to disinfection was investigated. *E. coli* is used as an indicator for fecal pollution for water quality monitoring, and was, thus, selected for this study. *E. coli* is used as an indicator for fecal pollution for water quality monitoring, and was, thus, selected for this study. To examine the effect of chemical-based differences between disinfectants on resistance development, two different chemical disinfectants, monochloramine and ferrate, were used. Bacterial physiological state and treatment conditions relevant to drinking water treatment and potable water reuse were also applied by cultivating *E. coli* populations to late-stationary phase in a nutrient-poor growth media, i.e., lake water media.

MATERIALS AND METHODS

E. coli Cultivation

Lake water media was sampled from the north wetland inlet of Ada Hayden Lake in Ames, IA (29 May 2018) and used for bacterial cultivation to simulate microbial growth conditions common in source surface waters for drinking water treatment. Characteristics of the sampled and amended lake water for bacterial cultivation have been described previously

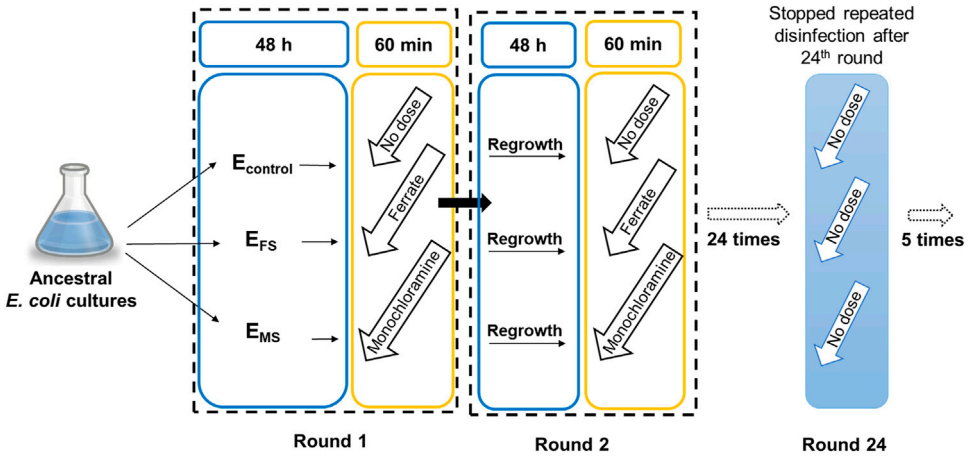

FIGURE 1 | Schematic of the experimental evolution experiment carried out to determine resistance development against disinfection. Experimental evolution of E_{MS}, E_{FS}, and $E_{control}$ under repeated monochloramine, ferrate and water treatment, respectively, every 48 h of bacterial growth.

(Daer et al., 2021). *E. coli* K-12 cultures were cultivated based on previous work (Daer et al., 2021). Briefly, *E. coli* K-12 cultures were cultivated for 24 h in modified Lysogeny broth (LB) media (tryptone 10 g/L, yeast extract 5 g/L, sodium chloride 5 g/L) at room temperature (23 ± 1.0 °C) under continuous shaking at 160 rpm. Cell pellets were harvested by centrifugation (14,000×*g*, 1 min), washed twice with sterile 10 mM sodium phosphate buffer (pH 7.0), and resuspended in amended lake water media. Cultures were grown for 24 h (160 rpm, 23 ± 1.0°C) to adapt to new growth conditions and were subsequently transferred to fresh amended lake water and regrown for 48 h to reach late stationary phase. This original population of *E. coli* is referred to as the ancestral culture since they represented the first generation of cells before they undergo multiple rounds of growth and disinfection.

Experimental Evolution and Disinfection

Ancestral cultures at late stationary phase were treated with either monochloramine, ferrate, or water to generate parallel monochloramine-stressed (E_{MS}), ferrate-stressed (E_{FS}), and control ($E_{control}$) cultures, respectively (**Figure 1**). Every 48 h of growth, the cultures were treated for 60 min and subsequently transferred to fresh amended lake water media to start a new growth round. As described in previous work (Daer et al., 2021), 2.5 mg/L as Cl_2 (35 μM) of sodium hypochlorite (8.25% w/v, commercial bleach) or 22.3 mg/L as Fe (400 μM) of potassium ferrate (97% purity, Element 26, League City, TX) were used to disinfect cultures for 60 min at each round. The water matrix composition pre-disinfection had a Cl_2:NH_3-N molar ratio below 1.0, which contributed to the generation of monochloramine due to hypochlorite addition. $E_{control}$ cultures were treated with an equivalent volume of sterile nanopure water for 60 min. Disinfection was quenched by transferring 2% (v/v) of cultures to fresh media of amended lake water. After 24 rounds of growth and disinfection, stressed cultures were regrown for an additional 5 rounds (48 h interval) without exposure to disinfectants. Changes in bacterial inactivation were monitored at select rounds. All experiments were conducted with at least three biological replicates.

Determination of *E. coli* Inactivation and Cellular Viability Post-disinfection

Culturable cell concentrations of E_{MS} and E_{FS} cultures were measured before and after disinfection to determine cellular inactivation. $E_{control}$ cultures were divided into three separate subcultures treated with either water, ferrate, or monochloramine (**Figure 1**). $E_{control}$ subcultures treated with water were transferred to the next round following 60 min, whereas the cellular inactivation of $E_{control}$ subcultures treated with ferrate and monochloramine was determined for comparison. Culturable cell concentrations were measured using heterotrophic plate counting following standard microbiological procedures (Reasoner, 2002). Cell inactivation was calculated based on the decrease in culturable cell concentration within 60 min of disinfection:

$$Inactivation = \log_{10}\left(\frac{N}{N_0}\right)$$

where N_0 and N represent culturable cell concentrations (CFU/ml) present initially and following disinfection, respectively.

Cellular viability based on intact membranes was assessed using LIVE/DEAD *Bac*light Bacterial Viability Kit (Molecular Probes, Eugene, OR). Briefly, E_{MS}, E_{FS}, and E_{conrol} were treated with monochloramine, ferrate, or water for 60 min, respectively. All cultures were subsequently pelleted, re-concentrated by 5 times in 0.9% (w/v) sodium chloride solution (pH 7.0 ± 0.1), and treated with LIVE/DEAD™ *BacLight* bacterial viability kit as per manufacturer's

instructions (L13152, Invitrogen, Carlsbad, CA). The percentage of live cells was determined for stressed and control cultures by microscopically analyzing at least 500 cells using a Zeiss Axio Imager fluorescence microscope (Zeiss, Oberkochen, Germany). All experiments were conducted in biological triplicates.

Whole Genome Sequencing of Ancestral and Evolved Cultures

Genomic DNA of ancestral (5 isolates) as well as E_{MS} (5 isolates), E_{FS} (3 isolates) and $E_{control}$ (3 isolates) cultures at the 32nd growth round was obtained and sequenced. Extraction of DNA was performed using GeneJET FFPE DNA Purification Kit as per manufacturer's instructions (ThermoFisher Scientific, Waltham MA). Sequencing was performed on Illumina HiSeq 3,000 using single-end layout mode and with a read length of 150 bases. The quality of the reads was inspected using FASTQC (v. 0.11.7) program (Simon, 2018) Demultiplexed files were mapped against the indexed reference genome of *E. coli* strain 1,223 (Genbank ID CP023383.1) using BWA (v 0.7.16) (Li and Durbin, 2010). Single nucleotide polymorphism (SNP) calling was performed using FreeBayes (v 1.0.2–38) and visualized using TASSEL (v 5.0) (Bradbury et al., 2007). Sequencing data has been made publicly available (Daer and Ikuma, 2021).

Effect of cAMP Levels on *E. coli* Culturability Following Monochloramine Disinfection

E_{MS} and $E_{control}$ cultures were treated with 1 mM of adenosine 3′, 5′-cyclic monophosphoric acid (cAMP) (Fisher Scientific, Waltham, MA) prior to disinfection to test whether changes in log inactivation were linked to culturability instead of cell viability. The preparation and dosage of cAMP were adapted from previous work by (Nosho et al., 2018). The pH of each culture was adjusted using 300 mM phosphate buffer (pH 8.0) to account for the increased acidity due to cAMP addition. Cultures with and without cAMP were subsequently treated with 2.5 mg/L as Cl_2 (35 μM) of monochloramine. Culturable cell concentrations were determined through heterotrophic plate counting following standard microbiological procedures (Reasoner, 2002). All experiments were conducted in biological triplicates.

Effect of Disinfection Frequency on Resistance Development

The time interval between each disinfection round was extended from 48 h to 96 and 144 h to examine the effect of monochloramine disinfection frequency on resistance development. The extension in the time interval to 96 and 144 h was selected because it was double and triple the 48 h growth interval, respectively. Cultures treated with monochloramine every 96 and 144 h are referred to as $E_{MS\text{-}96}$ and $E_{MS\text{-}144}$, respectively. Parallel control cultures ($E_{control\text{-}96}$ and $E_{control\text{-}144}$) were also grown and divided into two separate subcultures at specific disinfection rounds to receive either water or monochloramine. Within each round, cultures were transferred every 48 h to maintain a comparable water chemistry and physiological state (late stationary phase) as the 48 h cultures. At least 15 rounds of repeated disinfection were performed. All experiments were conducted in biological triplicates.

Statistical Analysis

Unless otherwise specified, all mean and standard errors were derived based on 3 biological replicates per treatment. Statistical significance was determined using two-tailed unpaired Student's t-tests in Excel. Unless otherwise specified, the confidence interval in all statistical tests was set at 95% with results being considered significantly different if the measured p-value was less than 0.05.

RESULTS

Effect of Repeated Disinfection on *E. coli* Inactivation and Resistance

E. coli were repeatedly disinfected with monochloramine (2.5 mg/L as Cl_2) or ferrate (22.3 mg/L as Fe) to examine changes in bacterial tolerance against both disinfectants. Inactivation of E_{MS} cultures significantly decreased from 4.0 ± 0.3-$\log_{10}$ to 2.0 ± 0.2-$\log_{10}$ within 12 monochloramine disinfection rounds (p-value = 0.01) (**Figure 2A**). In contrast, there was no significant change in the inactivation of E_{FS} cultures relative to ancestral (p-value = 0.3) and $E_{control}$ (p-value = 0.5) cultures after 21 rounds of ferrate disinfection (**Figure 2B**). These results indicate that repeated disinfection yielded bacterial populations that became less susceptible to monochloramine but not to ferrate.

A heritable and non-reversible increase in monochloramine tolerance could point to resistance development in the repeatedly treated cultures. Therefore, E_{MS} cultures were regrown for an additional five rounds without exposure to monochloramine to examine if cultures maintained the high monochloramine tolerance in the absence of repetitive disinfection. Cell inactivation of E_{MS} cultures in round 30 remained significantly lower than inactivation observed in ancestral (p-value = 0.00003) and $E_{control}$ (p-value = 0.0003) cultures by at least 1-$\log_{10}$ (**Figure 2A**). The genomes of ancestral and the evolved E_{MS} (round 32) and $E_{control}$ strains (round 32) were sequenced and compared against the *E. coli* 1,223 reference genome to examine for changes in genomic mutations due to repeated disinfection. No differences between the evolved, ancestral, and the reference genome were found (data available via public repository; Daer and Ikuma, 2021).

Effect of Repeated Disinfection on Cellular Viability of *E. coli*

As the loss of culturability does not necessarily indicate cell death, LIVE/DEAD staining was used as a culture-independent method based on membrane intactness to assess cell viability at select disinfection rounds. A significantly higher percentage of E_{MS} cells (60–80%) were viable (i.e., intact) compared to E_{FS} cells (<20%) (p-value = 0.0002) post-disinfection throughout the repeated

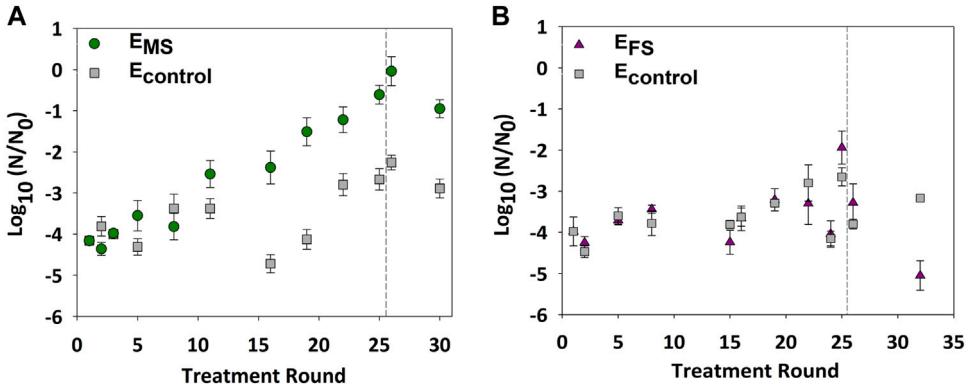

FIGURE 2 | *E. coli* $\log_{10}$ inactivation after multiple rounds of growth and disinfection with either 2.5 mg/L as Cl_2 of monochloramine or 11.2 mg/L as Fe of ferrate. **(A)** Bacterial inactivation of E_{MS} due to repeated monochloramine disinfection. **(B)** Bacterial inactivation of E_{FS} due to repeated ferrate disinfection. $E_{control}$ cultures were not repeatedly treated; separate aliquots of the $E_{control}$ were obtained at select rounds and treated for comparison. Rounds in which stressed cultures were regrown without exposure to disinfection are presented after the dashed line. Each data point represents an average of at least triplicate samples. Error bars represent one standard error of at least triplicate samples. Initial cell concentration (N_0) ~ 10^8 CFU/ml; pH = 8.4; room temperature.

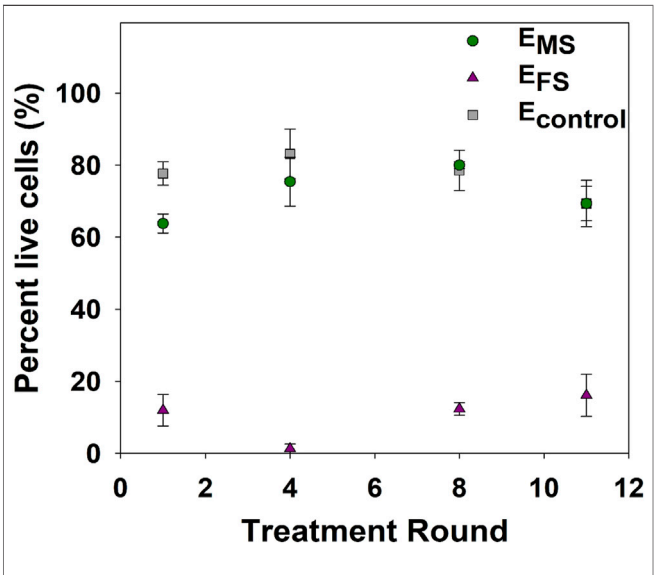

FIGURE 3 | Percentage of viable cells after 60 min of treatment with 11.2 mg/L as Fe of ferrate (E_{FS}) or 2.5 mg/L as Cl_2 of monochloramine (E_{MS}), or water ($E_{control}$). Each data point represents an average of at least triplicate samples. Error bars represent one standard error of at least triplicate samples.

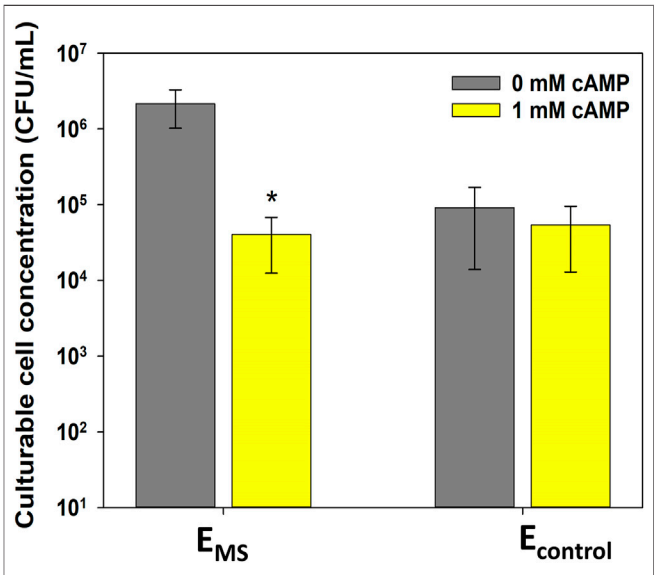

FIGURE 4 | Effect of cAMP addition on culturable cell concentration of $E_{control}$ and E_{MS} cultures after 60 min of monochloramine treatment at the 16th disinfection round. Error bars represent one standard deviation of at least triplicate samples. Asterisks (*) indicate statistical significance (p-value < 0.05) compared to 0 mM cAMP addition.

disinfection cycles (**Figure 3**), pointing to the formation of VBNC cells in response to monochloramine disinfection. The viability of E_{MS} and E_{FS} cultures did not significantly change in response to repeated monochloramine and ferrate disinfection, respectively.

Influence of cAMP Concentration on VBNC Induction in Evolved Cultures

The effect of exogenous cAMP addition on the culturability of evolved E_{MS} and $E_{control}$ cultures after 60 min of monochloramine treatment was investigated. Culturable cell concentration in E_{MS} cultures pretreated with cAMP was lower by 1.8 ± 0.4-$\log_{10}$ compared to E_{MS} cultures without cAMP pretreatment (p-value = 0.0006, **Figure 4**). The percentage of viable cells in E_{MS} with and without cAMP addition was not significantly different (p-value > 0.05, **Supplementary Figure S2**), suggesting that the decrease in culturable cell concentrations in response to cAMP addition was not caused by losses in cell viability. Interestingly, cAMP addition did not affect culturable cell concentrations of

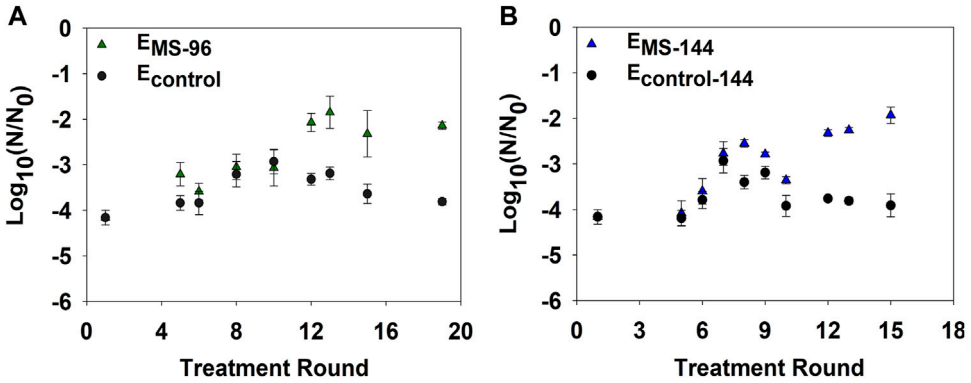

FIGURE 5 | *E. coli* $\log_{10}$ inactivation after multiple rounds of growth and disinfection with 2.5 mg/L as Cl_2 of monochloramine. **(A)** Bacterial inactivation of E_{MS-96} due to repeated monochloramine disinfection every 96 h. **(B)** Bacterial inactivation E_{MS-144} due to repeated monochloramine disinfection every 144 h. $E_{control-96}$ and $E_{control-144}$ were not repeatedly treated; subcultures of each were obtained and treated at select treatment rounds for comparison. Error bars represent one standard error of at least triplicate samples. Initial cell concentration (N_0) ~10^8 CFU/ml; pH = 8.4; room temperature.

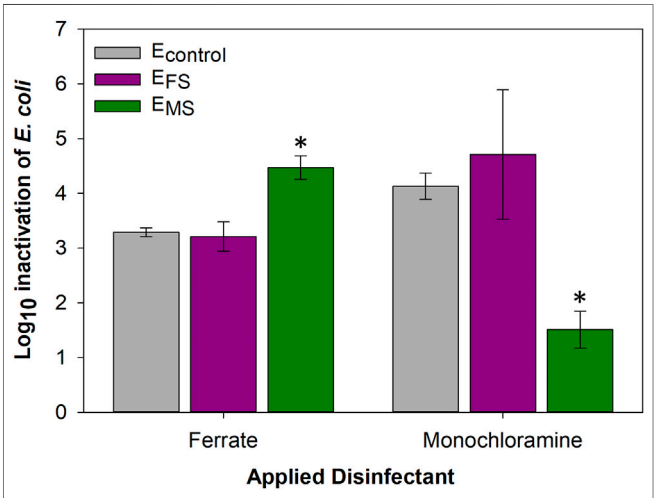

FIGURE 6 | *E. coli* $\log_{10}$ inactivation of controls and evolved (E_{FS} and E_{MS}) cultures cross-disinfected with ferrate or monochloramine at the 18th disinfection round. Single asterisks (*) represent statistical significance (p-value < 0.05) compared to $E_{control}$ cultures treated with the respective disinfectant. Error bars represent one standard deviation of at least triplicate samples.

$E_{control}$ cultures following monochloramine treatment (**Figure 4**). This observation suggests that cAMP addition to controls did not appear to cause additional cells to lose culturability and become VBNC. In addition, there was no statistical difference between culturable cell concentrations in control cultures treated with or without cAMP and E_{MS} cultures treated with cAMP (**Figure 4**). Therefore, the significant increase in cell culturability caused by repeated monochloramine disinfection could be reversed back to baseline levels in the presence of higher cAMP concentrations.

Effect of Monochloramine Disinfection Frequency on Resistance Development

To examine if a decrease in disinfection frequency could hinder resistance development, we incrementally extended the time interval between each monochloramine disinfection round from 48 to 96 and 144 h, thus generating E_{MS-96} and E_{MS-144} cultures, respectively. Inactivation of E_{MS-96} cultures significantly decreased by 2-$\log_{10}$ compared to ancestral (p-value = 4×10^{-5}) and $E_{control}$ (p-value = 0.001) after 12 rounds of disinfection (**Figure 5A**). A significant drop in inactivation of E_{MS-144} was also observed following 12 monochloramine disinfection rounds compared to ancestral (p-value = 3×10^{-6}) and $E_{control}$ (p-value = 7×10^{-8}) (**Figure 5B**).

Susceptibility of Stressed *E. coli* Cultures to Cross Disinfection

E_{MS} and E_{FS} cultures were cross-disinfected with ferrate and monochloramine, respectively, to determine if evolutionary adaptations triggered by frequent disinfection could protect cells against exposure to the other disinfectant. The susceptibility of E_{MS} cultures to ferrate was higher by 2.96 ± 0.40-$\log_{10}$ compared to monochloramine (p-value = 0.0009) (**Figure 6**). In fact, E_{MS} cultures were found to be more susceptible to ferrate compared to control and E_{FS} cells (p-value < 0.05). In contrast, E_{FS} cultures were inactivated to similar extents by ferrate and monochloramine (p-value > 0.05) (**Figure 6**).

DISCUSSION

In this work, repeated growth and disinfection of *E. coli* cultures enhanced bacterial tolerance against monochloramine (**Figure 2A**), but not ferrate (**Figure 2B**). Results also showed that removing repeated exposure to monochloramine after 25 disinfection rounds did not

restore the E_{MS} tolerance against monochloramine to levels observed in ancestral cultures (**Figure 2A**). Since monochloramine tolerance did not reverse back to ancestral susceptibility levels, repeated monochloramine disinfection likely drove resistance in *E. coli* cultures against monochloramine. These results are in line with earlier studies that reported chlorine resistance in *E. coli* following deliberate and repeated exposure to the disinfectant (Farkas-Himsley, 1964; Leyval et al., 1984; Gundlach and Winter 2014). In previous studies, E. *coli* cultures that were either isolated from source water routinely treated with chlorine (Farkas-Himsley, 1964) or repeatedly exposed to free chlorine (Leyval et al., 1984; Gundlach and Winter 2014) tolerated increasingly higher chlorine dosages. The underlying mechanisms contributing to resistance against chlorine or oxidative stress-inducing conditions remain not well understood (Du et al., 2015; da Cruz Nizer et al., 2020; Wang et al., 2020). Adaptive laboratory evolution of *E. coli* in the presence of hypochlorite (Gundlach and Winter 2014) and hydrogen peroxide (Anand et al., 2020) showed that cells acquired resistance due to evolutionary processes that favored the constitutive expression of OxyR-regulated genes. In these studies, the evolutionary adaptations were attributed to either genomic mutations (Anand et al., 2020) or epigenetic changes (Gundlach and Winter 2014). To examine if the evolved resistance against monochloramine was due to genomic mutations in this study, we sequenced the genomes of the ancestral and the evolved E_{MS} (round 32) and $E_{control}$ strains (round 32) and compared them against the *E. coli* 1,223 reference genome. The lack of notable mutations in E_{MS} cultures suggests that the evolutionary adaptions could be due to epigenetic changes or the constitutive expression of stress-related responses.

Mechanisms that trigger tolerance or resistance, or lack thereof, against disinfectants remain poorly understood, although a handful of studies have suggested the potential involvement of oxidative stress responses (Dukan and Touati, 1996; Berry et al., 2010; Gundlach and Winter 2014). As highlighted previously (Gundlach and Winter 2014), showed that *E. coli* populations resisted frequent and incremental increases in hypochlorite dosage due to elevated OxyR-dependent oxidative stress responses. On the other hand, Ikai et al. (2013) showed that frequent disinfection using fast-acting oxidants involving hydroxyl radicals did not alter *E. coli* susceptibility following 40 exposure cycles. It was hypothesized that the fast-acting hydroxyl radical indiscriminately targeted cellular structures and metabolic pathways, preventing the evolution of resistance (Ikai et al., 2013). Similarly, ferrate exhibits a faster mode of disinfection compared to monochloramine (Ramseier et al., 2011; Daer et al., 2021). Therefore, the fast action of ferrate and ferrate-derived reactive oxygen species may have hindered adaptive processes against the disinfectant, leading to the lack of resistance development against ferrate. Additionally, we have previously shown that *E. coli* cultures treated with ferrate resulted in a different gene expression profile than cultures treated with monochloramine, suggesting that the disinfectants trigger different cellular responses (Daer et al., 2021).

An analysis of cellular viability post-disinfection showed that ferrate resulted in a significant loss in viability compared to monochloramine (**Figure 3**). In line with previous studies (Ramseier et al., 2011; Linklater, 2017; Daer et al., 2021), ferrate consistently inactivated E_{FS} cultures in each disinfection round by disrupting the cell envelope, as evidenced by the significantly higher percentage of damaged cells after each disinfection episode (**Figure 3**). On the other hand, the observed differences between LIVE/DEAD results and culturable cell concentrations (**Figure 2A** and **Figure 3**) of E_{MS} cultures indicate that monochloramine induced a fraction of *E. coli* into the viable but nonculturable (VBNC) state in the initial disinfection rounds. The VBNC fraction of E_{MS} cultures likely decreased after 12 + disinfection rounds with more cells retaining culturability as evidenced by the increase in culturable cell concentrations (**Figure 2A**). As such, the disinfectants' mode of action and adaptive responses against disinfection may have contributed to the observed differences in the inactivation trend over the course of repeated disinfection rounds.

VBNC induction in response to UV and chlorine-based disinfectants has been observed (Ben Said et al., 2010; Lin et al., 2017; Chen et al., 2018; Zhang et al., 2018). Although VBNC cells are viable and intact, they exhibit limited metabolism compared to culturable counterparts (Zhao et al., 2016; Lin et al., 2017; Ye et al., 2020). By reducing essential metabolic processes and becoming dormant, VBNC cells can withstand various stressors, including oxidative stress (Chen et al., 2018; Liao et al., 2019), prolonged starvation (Liu et al., 2009), and extreme environments (Boaretti et al., 2003), and may resuscitate if favorable growth conditions are restored. Similarly, VBNC induction may be a survival strategy adopted by a fraction of the *E. coli* population in response to monochloramine disinfection. As opposed to ferrate, cells' ability to withstand monochloramine while retaining viability may have driven resistance development against repeated monochloramine disinfection.

Following 12 + disinfection rounds, the observed increase in cell culturability within the population post-disinfection was not likely caused by VBNC cells resuscitating and becoming culturable within the 60 min disinfection timeframe. Bacterial resuscitation from VBNC states has been shown to be slow and dependent on inducers' presence in the resuscitation media (Liu et al., 2009). The change in culturable cell concentrations over the exposure time was examined to confirm the absence of cell resuscitation. As shown in **Supplementary Figure S1**, the loss of culturable cell concentrations over the disinfection exposure time (60 min) was gradual and significantly smaller in E_{MS} cultures compared to ancestral and $E_{control}$. These observations indicate that the percentage of the *E. coli* cells likely to enter the VBNC state decreased following multiple rounds of monochloramine disinfection. VBNC-induced state in *E. coli* by monochloramine may be a regulated process which is also influenced through repeated exposure to the disinfectant. **Figure 7** schematically illustrates the potential shift in the fraction of *E. coli* population that enter VBNC due to multiple

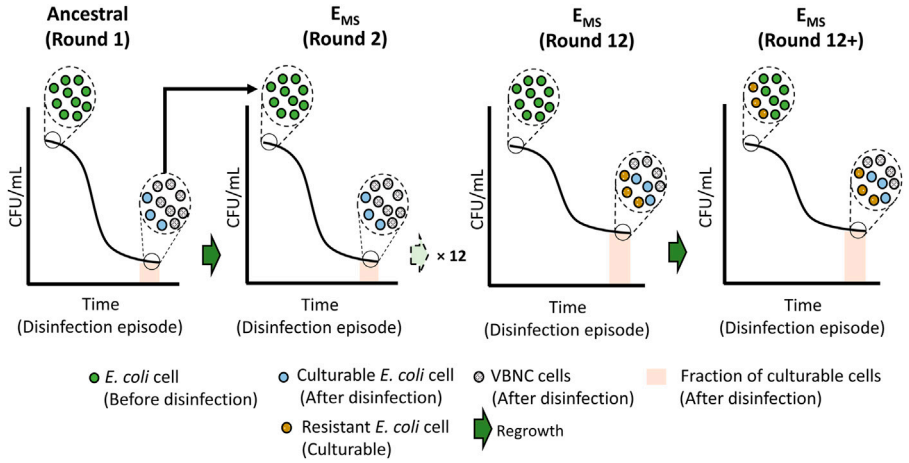

FIGURE 7 | Schematic demonstrating the potential emergence of resistant cells and the shift in the fraction of VBNC and culturable cells post-disinfection in response to multiple rounds of monochloramine disinfection.

episodes of monochloramine disinfection, leading to the observed resistance pattern against monochloramine.

An understanding of the molecular pathways that regulate entry into the VBNC state remains elusive, although critical to better understand this study's findings (Boaretti et al., 2003; Li et al., 2014; Pinto et al., 2015; Dong et al., 2020). Recent literature suggests that interrelated mechanisms involving the major stress regulator (RpoS), guanosine pentaphosphate [(p) ppGpp], LysR-type transcriptional factor (OxyR), cAMP, and cAMP-receptor protein (CRP) contribute to the formation of VBNC cells (Boaretti et al., 2003). Boaretti et al. (2003) showed that *E. coli* strains with increased (p) ppGpp production did not readily lose culturability and enter VBNC state following prolonged starvation. Nosho et al. (2018) reported that starvation caused wild-type cells to lose culturability (i.e., enter a VBNC state) but not mutant *E. coli* strains that lacked cAMP or CRP production. Furthermore, *E. coli* strains with decreased cAMP levels and cAMP-CRP activity conferred high resistance to hypochlorous acid due to the accumulation of cellular RpoS (Barth et al., 2009). Hence, cAMP and the cAMP-CRP regulatory pathway could play a role in VBNC induction and resistance to monochloramine. We hypothesized that a drop in cellular cAMP concentrations in E_{MS} cultures may be the underlying cause for the lower occurrence of the cAMP-CRP-induced VBNC state and, in turn, the simultaneous increase in bacterial culturability. In other words, E_{MS} cultures may have adapted to repeated monochloramine disinfection by regulating cAMP levels and minimizing the likelihood of the monochloramine-induced VBNC state. Although transcriptomic analysis was beyond the scope of this study, it is the next step for identifying the regulatory pathways and genes involved in the drop of cAMP levels due to frequent monochloramine treatment.

Given the hypothesized relationship between cellular cAMP concentration, entry into the VBNC state, and monochloramine resistance, we examined the effect of cAMP levels on culturability of evolved *E. coli* cultures. As results show, evolved E_{MS} cultures that were pretreated with cAMP were significantly less culturable following monochloramine treatment compared to E_{MS} cultures that were not pretreated with cAMP (**Figure 4**). In addition, presence of exogenous cAMP during disinfection changed the culturability of E_{MS} but not $E_{control}$, indicating that repeated monochloramine disinfection triggered changes in the cAMP-CRP system in resistant cells, possibly through decreased intracellular cAMP concentrations.

Intracellular cAMP levels are dynamically regulated through multiple, complex mechanisms that involve the sugar phosphotransferase system (PTS), cellular ATP levels, efflux proteins, adenyl cyclases, and phosphodiesterases (Green et al., 2014). Higher cAMP levels have been linked to nutrient and environmental stress in bacteria (Shimizu, 2013). In fact, results from our previous study indicate that a one-time monochloramine dose (i.e., disinfection round 1) induces the expression of PTS-related genes and downregulates genes encoding cAMP-relevant efflux proteins such as *tolC* in late stationary phase *E. coli* cells (Daer et al., 2021), both of which could lead to increased intracellular cAMP levels (Postma et al., 1993; Green et al., 2014) in the ancestral and $E_{control}$ cultures receiving only single disinfectant doses without repetition. Though more work is needed to determine the mechanisms, our observations suggest that repeated monochloramine disinfection triggered evolutionary processes that led to lower intracellular cAMP concentrations in resistant E_{MS} cells.

Another key question is whether a lower disinfection frequency could prevent the emergence of bacterial resistance against monochloramine. Recurring stressors can facilitate resistance development in bacteria by increasing the frequency of stress-induced mutagenesis, epigenetic changes, or selection of favorable adaptations (Gundlach and Winter 2014; LaCroix et al., 2015; Lukačišinová et al., 2017; Anand et al., 2020). However, stress-induced adaptations confer a fitness cost that discourages resistance evolution under favorable growth conditions in which growth stressors are absent or not frequent (Björkholm et al., 2001; Day, 2016; van den Bergh et al., 2016). For example, bacterial populations exposed to a disinfectant every 100

generations may trigger resistance, whereas exposure every 1,000 generations may not be frequent enough to drive the mutagenesis or selection of adaptive traits. In a potable water reuse context, time between disinfection events (i.e., disinfection frequency) could be controlled by the size of the environmental buffer (for IPR) or the length of retention times within pipes and treatment trains (for DPR). However, our results indicate that monochloramine resistance occurred despite the reduction in disinfection frequency (**Figure 5A**). As mentioned above, frequent monochloramine disinfection (every 48 h) drove monochloramine resistance by increasing the fraction of cells that retained culturability post-disinfection through mechanisms involving cAMP (**Figure 4**). Similarly, culturable cell concentrations in $E_{MS\text{-}96}$ and $E_{MS\text{-}144}$ cultures pretreated with cAMP were lower by 1.5 ± 0.5-$\log_{10}$ and 0.5 ± 0.4-$\log_{10}$ compared to E_{MS} cultures without cAMP pre-treatment, respectively (**Supplementary Figure S3, S4**). As such, the relative increase in culturable cell concentrations upon monochloramine exposure in evolved populations of *E. coli* appears to be regulated by cAMP-dependent pathways even when the intervals between disinfection episodes were varied. These observations further suggest that despite infrequent disinfection, similar physiological responses evolved within the bacterial populations, leading to resistance against repeated monochloramine disinfection.

The recurrence of other growth-related stressors during each growth round, i.e., late stationary phase, may explain monochloramine resistance development despite infrequent disinfection. During late stationary phase, bacterial cultures grow in harsher conditions due to nutrient deprivation, resource competition, and other growth-related stressors. For example, Lisle et al. (1998) observed that phenotypic tolerance against chlorine increased in *E. coli* cultures that were carbon-starved. Du et al. (2015) found that starved *E. coli* cultures grown solely in phosphate-buffered saline solutions resisted monochloramine treatment by triggering proteomic adaptations against starvation and oxidative stress. In a previous study, we noted the elevated expression levels of general stress responses pre-disinfection due to late-stationary phase growth stressors, which could influence bacterial stress responses following monochloramine treatment (Daer et al., 2021). Frequent regrowth into late stationary phase and recurrence of stationary phase stress factors may have imposed an additional selective pressure for monochloramine resistance. Finished water quality in water reuse is oligotrophic due to the low concentration of nutrients and organic matter post-treatment, pointing to the likelihood that bacterial cells present in the water would have late stationary phase-like physiologies. Therefore, growth-related stressors in potable water reuse may further exacerbate monochloramine resistance.

Lastly, E_{MS} and E_{FS} cultures were cross-disinfected with ferrate and monochloramine, respectively, to determine if evolutionary adaptations triggered by frequent disinfection could protect cells against exposure to the opposite disinfectant. Cross-disinfection results showed that E_{MS} cultures were significantly more susceptible to ferrate compared to monochloramine (**Figure 6**), suggesting that the cellular adaptations triggered by the repeated monochloramine treatments did not enhance tolerance against ferrate. Ferrate can react more strongly with cell membranes compared to chlorine-based disinfectants and has been shown to exhibit faster disinfection kinetics (Daer et al., 2021). Ferrate also possesses a higher oxidation potential (E^0 = 2.2V, acidic media (Wood, 1958)) than monochloramine (E^0 = 1.5V, pH 0 (Rajasekharan et al., 2007)), which could result in different oxidative reactions in bacterial cells. Similarly, previous studies have shown that a greater percentage of *E. coli* cells lost cell integrity and, in turn, viability due to ferrate exposure compared to hypochlorite (Linklater, 2017). Ramseier et al. (2011) noted that the damage to the membranes of drinking water bacterial cells was two orders of magnitude higher due to ferrate ($\sim 9 \times 10^{-3}$ L/mg×min) compared to monochloramine ($\sim 4 \times 10^{-5}$ L/mg×min) treatment. Furthermore, bacterial mixtures from the chlorine-resistant genera, such as *Mycobacterium* and *Bacillus*, were inactivated by more than 99.8% using ferrate, despite their durable cell wall structure (Gombos et al., 2012). Therefore, we speculate that ferrate and its intermediates indiscriminately and rapidly (within minutes) oxidize cellular components, thereby causing irreversible cellular damage and interfering with the evolution of adaptations against disinfection. As such, intracellular adaptations against repeated monochloramine treatment did not protect adapted cells against ferrate-driven oxidation of the membrane and its constituents.

Environmental Implications

As water utilities move towards shorter urban water cycles, microbial resistance and decreased susceptibility to conventional disinfectants pose new challenges to disinfection. This study explored the potential development of bacterial resistance in response to repeated disinfection that may occur in potable water reuse scenarios. Our observations suggest there is a potential risk in microbial resistance developing against repeated disinfection that is mediated by changes in the culturability of bacterial cells. At the molecular level, epigenetic changes or the constitutive expression of stress-related responses in *E. coli* may have triggered the enhanced bacterial response against frequent monochloramine disinfection. However, the lack of resistance observed under repeated ferrate disinfection also suggests that using strong oxidants with high reactivity for primary disinfection may be advantageous for pathogen eradication (resistant or otherwise) compared to slower-acting chlorine-based disinfection. Since the development of disinfection resistance is highly undesirable, AWT trains used in potable water reuse will need to implement appropriate disinfectants and measures that minimize the formation of resistant organisms post-disinfection. Disinfectants that remove pathogens while preventing pathogen resistance are crucial in potable water reuse systems. Based on this study, the reactivity of a disinfectant may be an important criterion to consider for the selection of effective and alternative primary disinfectants in potable water reuse, even if slower-acting chlorine-based compounds are necessary as residual disinfectants. Furthermore, this work showed that bacterial physiological responses, such as entry into the VBNC state, could facilitate the long-term emergence of bacterial resistance in

response to repeated exposure to monochloramine. Future work is needed to unravel the molecular pathways that govern and regulate those physiological responses among various emerging pathogens of concern. Overall, the findings of this study are important for re-evaluating and re-designing current and future water disinfection schemes to protect public health even as climate and water use patterns continue to evolve and change.

DATA AVAILABILITY STATEMENT

The dataset accompanying this study has been made publicly available on Figshare. Data can be accessed using the following link: https://doi.org/10.6084/m9.figshare.14785845.v1.

AUTHOR CONTRIBUTIONS

SD conducted the experiments, analyzed the data, and wrote the manuscript. ER and JR conducted the experiments. KI designed the experiments, modified the manuscript and provided feedback. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2022.855224/full#supplementary-material

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