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### Keywords

Disinfection; Ferrate; Monochloramine; RNA-seq; Transcriptomics; VBNC

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## Highlight

- Evidence of VBNC induction noted due to monochloramine disinfection but not ferrate
- *E. coli* transcriptome pre and post disinfection was examined using RNA-seq
- More genes were upregulated due to monochloramine treatment compared to ferrate
- Monochloramine downregulated transcription, translation and stress response genes

Journal Pre-proof

## Effect of ferrate and monochloramine disinfection on the physiological and transcriptomic response of *Escherichia coli* at late stationary phase

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### Abstract

Biological mechanisms of disinfection not only vary by disinfectant but also remain not well understood. We investigated the physiological and transcriptomic response of *Escherichia coli* at late stationary phase to ferrate and monochloramine in amended lake water. Although ferrate and monochloramine treatments similarly reduced culturable cell concentrations by 3-log<sub>10</sub>, 64% and 11% of treated cells were viable following monochloramine and ferrate treatment, respectively. This observed induction of viable but non-culturable (VBNC) state following monochloramine treatment but not ferrate is attributed to slower monochloramine disinfection kinetics (by 2.8 times) compared to ferrate. Transcriptomic analysis of *E. coli* at 15 min of exposure revealed that 3 times as many genes related to translation and transcription were downregulated by monochloramine compared to ferrate, suggesting that monochloramine treatment may be inducing VBNC through reduced protein synthesis and metabolism. Downregulation of universal stress response genes (*rpoS*, *uspA*) was attributed to growth-related physiological stressors during late stationary phase which may have contributed to the elevated expression levels of general stress responses pre-

disinfection and, subsequently, their significant downregulation post-disinfection. Both disinfectants upregulated oxidative stress response genes (*trxC*, *grxA*, *soxS*), although levels of upregulation were time sensitive. This work shows that bacterial inactivation responses to disinfectants is mediated by complex molecular and growth-related responses.

**Keywords:** Disinfection, Ferrate, Monochloramine, RNA-seq, Transcriptomics, VBNC

## 1. Introduction

Inactivation of biological contaminants using disinfection has led to a global reduction in the spread of waterborne diseases. However, alternative water management strategies such as potable water reuse pose new challenges for disinfection (Chaudhry et al., 2017). Though conventional methods of disinfection, such as chlorine, ozone, monochloramine, and ultraviolet radiation, are effective in many cases, these methods still present challenges to water treatment. For example, chlorine and chloramine can generate carcinogenic disinfectant byproducts (DBPs) such as trihalomethanes, haloacetic acids, and haloacetonitriles through reactions with source water organic matter (Reckhow et al., 1990; Yang et al., 2007). Therefore, alternative disinfection processes are needed for the continued development of drinking water treatment. Ferrate is being considered as a new disinfectant due to its multipurpose capabilities that include oxidation, coagulation, and disinfection (Jiang et al., 2006; Sharma et al., 2005). Furthermore, compared to other oxidants, the generation of DBPs using ferrate is relatively low, rendering it an environmentally friendly alternative (Sharma et al., 2015).

Understanding the pathogens' biological response to disinfectants is necessary for improved prediction of disinfection effectiveness and process innovation. However, the interactions that occur between various disinfectants and cellular components or processes during inactivation remain unclear (Cho et al., 2010). Investigations of bacterial inactivation using oxidant-based disinfectants (e.g. ozone, chlorine, hypochlorite, hydrogen peroxide) suggest that cells may be inactivated through oxidative damage of cellular constituents (Dukan et al., 1996). Assessments using knock-out mutants (Dukan and Touati, 1996), advanced microscopy (Ofori et al., 2017), and protein or enzymatic assays (Cho et al., 2010) suggest that disruption to the cytoplasmic membrane, protein degradation, or damage to DNA are possible inactivation pathways. It is important to note that bacterial inactivation has traditionally been measured by assessing the loss of culturability through heterotrophic plate counting (Rice et al., 2012). However, chlorination and UV irradiation have been shown to induce viable but non culturable (VBNC) state among several bacterial species, including *E. coli*, *Pseudomonas* spp., and *Aeromonas* spp. (Chen et al., 2019, 2018; Guo et al., 2019; Lin et al., 2017; Zhang et al., 2015).

In-depth study of the bacterial transcriptome recently became possible through advancements in high-throughput screening of gene expression such as RNA-seq. RNA-seq is a powerful next generation sequencing tool that can analyze global gene expression more comprehensively compared to microarrays (Zhao et al., 2014). Thus far, a few studies explored the bacterial transcriptomic response to disinfectants including free chlorine (Ceragioli et al., 2010; Wang et al., 2010, 2009; Ye et al., 2020), monochloramine (Berry et al., 2010; Holder et al., 2013), peracetic acid and hydrogen

peroxide (Ceragioli et al., 2010; Small et al., 2007). The comparative studies showed that exposure to disinfectants influenced similar biological functions overall, but also led to transcriptomic responses that were disinfectant-specific in *Pseudomonas aeruginosa* (Small et al., 2007) and *Bacillus cereus* (Ceragioli et al., 2010). Many of these studies used bacterial cells grown in rich media and exposed to disinfectants in phosphate buffered saline, which is chemically less complex compared to environmental source waters. Although such conditions allow for experimental consistency across studies, they do not explain cellular responses to disinfectants at physiological states and growth conditions realistic to drinking water treatment. Understanding of the biological mechanisms triggered by alternative disinfectants such as ferrate (Fe(VI)), is even more limited (Sharma et al., 2015). Ferrate disinfection is a relatively fast reaction that can inactivate cells within 10 minutes, however, its mechanism of interaction with bacterial cells is still largely unknown (Fan et al., 2018; Gombos et al., 2012; Hu et al., 2012; Kazama, 1994; Ramseier et al., 2011).

The goal of this study was to evaluate bacterial inactivation and the biological response of *Escherichia coli*, a model bacterium, at both the physiological and molecular levels to two different disinfectants: potassium ferrate and the more commonly applied, monochloramine. Bacteria were cultivated in lake water until late stationary phase to better understand cellular responses under bacterial physiological conditions more relevant to nutrient limited environmental systems. Global gene expression analysis using RNA-seq was performed to examine molecular responses since the activity of biological functions can be predicted from the level of transcribed genes in a cell. An improved understanding of the mechanisms involving bacterial inactivation could

enhance current disinfection practices as well as future and alternative disinfection strategies.

## **2. Materials and methods**

### **2.1. Growth media matrix and characteristics**

Lake water was sampled from the north wetland inlet of Ada Hayden Lake in Ames, IA (May 29, 2018). Briefly, Ada Hayden Lake is characterized as being moderately oligotrophic with moderate levels of TOC, slightly alkaline pH, and trace amounts of ammonia and nitrates (Table S1). Lake water was used to simulate microbial growth conditions common in source surface waters for drinking water treatment. Lake water was filtered through glass microfiber membranes followed by filtration using 0.45  $\mu\text{m}$  pore size cellulose membranes to remove suspended solids. Filtered lake water was then stored at 4°C until use. For bacterial cultivation, lake water was amended with 100 mg/L yeast extract and 1 mM phosphate buffer, and autoclaved. Amending lake water with yeast extract was necessary to support bacterial growth to cell concentration levels that are adequate for quantification and RNA extraction. All chemicals used were ACS grade and obtained from Fisher Scientific (Waltham, MA) unless otherwise specified.

### **2.2. Cultivation and growth**

Three independent biological replicates of *E. coli* K-12 cultures were grown in modified Lysogeny broth (LB) media (tryptone 10 g/L, yeast extract 5 g/L, sodium chloride 5 g/L) at room temperature (22-23°C) with continuous shaking at 160 rpm for 24 h. Cells were harvested by centrifugation (14,000  $\times g$ , 1 min) and pellets were washed twice using 10 mM sodium phosphate buffer (pH 7.0) and subsequently resuspended in amended lake water media. Cultures were grown in amended lake water for 24 h to allow them to



adapt to new growth conditions, and then transferred to fresh amended lake water media and grown for 48 h with shaking at 160 rpm at room temperature to reach late stationary phase (Fig. S1). This growth phase better resembles bacterial cell conditions observed in oligotrophic surface water (Navarro Llorens et al., 2010). *E. coli* growth also altered the overall water chemistry (Table S1). The final pH of the cultures immediately before disinfection was approximately 8.4. These characteristics represent the water chemistry immediately pre-disinfection.

### **2.3. *E. coli* disinfection kinetics and disinfectant decay in amended lake water**

Following 48 h growth, cultures were treated with sodium hypochlorite (8.25% w/v, commercial bleach) for monochloramine generation or potassium ferrate (97% purity, Element 26, League City, TX). At least three independent cultures were exposed to hypochlorite doses of 2.5 and 4.6 mg/L as Cl<sub>2</sub> (35 and 65 μM, respectively) or ferrate doses of 11.2 and 22.3 mg/L as Fe (200 and 400 μM, respectively) of ferrate. These doses were selected based on preliminary data to yield between 1 and 6 log<sub>10</sub> levels of *E. coli* inactivation levels within 60 min of exposure (Fig. S2). The water matrix immediately after disinfection had a Cl<sub>2</sub>:NH<sub>3</sub>-N molar ratio of less than 1.0 with the applied hypochlorite concentrations (Table S1), indicating that monochloramine was the dominant Cl<sub>2</sub> species present. For all cell inactivation measurements, cells were enumerated by heterotrophic plate counting following standard microbiological procedures. Aliquots of cultures were collected 0 minutes (before exposure) and after 3, 7, 15, 20, 45 and 60 min of exposure and serially diluted in LB to quench the reaction for plate counting. Losses in culturable cells was considered inactivation. Further details

of the disinfection experiments are provided in the SI. Unless otherwise specified, all experiments were conducted in biological triplicates.

#### **2.4. Test of bacterial viability**

Monochloramine-stressed, ferrate-stressed and control cultures were pelleted, re-concentrated by 5 times in 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). Percentage of total cells that are live 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.

#### **2.5. Time course exposure to disinfectants and RNA extraction for transcriptomic profiling**

Three independent biological replicates of *E. coli* cultures were treated with either 2.5 mg/L as Cl<sub>2</sub> of monochloramine or 22.3 mg/L as Fe of ferrate. The doses were selected so that sufficient inactivation response (approximately 3-log<sub>10</sub> inactivation) was measured while allowing for a residual population of surviving cells for harvesting quality RNA. Control experiments followed the same procedure using sterile water instead of the disinfectants. Reactions were quenched with LB and cells were harvested through centrifugation at 0 minutes (before exposure) and after 5, 15, 30, and 60 min of exposure, washed with 10 mM phosphate buffer (pH 7.0), and subsequently treated with RNAlater Stabilization Solution (Invitrogen, Carlsbad, CA). Samples were kept at 4°C for 48 h before storing at -20°C as cell pellets. RNA was extracted using a RNeasy Mini Kit (Qiagen Inc., Valencia, CA) with DNase I digestion following the manufacturer's procedure.

## 2.6. RNA-seq analysis of *E. coli* pre- and post-disinfection

RNA extracted from cells exposed to monochloramine, ferrate or water at 0 and 15 min (section 2.5) were used for subsequent RNA-seq analysis at the Iowa State University DNA Facility. Detailed information about the mRNA sample preparation for RNA-seq is in Text S1. The samples were sequenced on an Illumina HiSeq 3000 system using a paired ended sequencing option with 150 base pair read lengths. Sequencing data was processed through the Genome Informatics Facility at Iowa State University. Detailed information about the quality of reads and data processing steps is provided in Text S1. The DESeq2 R-package was used for the normalization and calculation of differential expression levels (Love et al., 2014). Genes were considered differentially expressed (DE) if they had a False Discovery Rate (FDR)  $\leq 0.001$  and a  $\log_2$  fold-change (FC) above or below 2.0. For meaningful biological interpretation, differentially expressed genes were annotated according to functional classes using Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (Huang et al., 2009a, 2009b). Gene ontology (GO) functional enrichment analysis was carried out for sets of differentially expressed genes to assess if biological functions were enriched or over-represented (Tipney and Hunter, 2010). GO terms with a fold enrichment above 1.0 and  $p < 0.1$  (Benjamini-Hochberg test) were considered significantly enriched. Transcriptome data has been deposited at Gene Expression Omnibus under accession number GSE126176.

## 2.7. Quantitative reverse transcription PCR (qRT-PCR) of selected genes

The transcript amounts of six genes (*grxA*, *trxC*, *soxS*, *recA*, *rpoS*, and *gshB*) were determined by two-step qRT-PCR. Genes related to stress responses (*recA* involved in DNA repair, *rpoS* involved in the general stress response, and *gshB* involved in

glutathione biosynthesis) (Battesti et al., 2011; Carmel-Harel and Storz, 2000; Lusetti and Cox, 2002) as well as other important genes based on the RNA-seq results (*grxA*, *trxC*, and *soxS*) were selected for analysis. Total RNA samples extracted from *E. coli* cells obtained at 0, 5, 15, 30, and 60 min of disinfectant exposure were analyzed. Detailed information about the qRT-PCR protocol is in Text S1. The transcript copies of each target gene were normalized to that of the 16S rRNA gene. Fold change at each exposure time point was calculated by comparing to the time point immediately before treatment.

## 2.8. Statistical analysis

Unless otherwise specified, statistical significance was determined using two-tailed Student's t-tests. The type of Student's t-test was selected depending on whether cultures were biologically matched (paired t-test) or not (unpaired t-test). 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.

## 3. Results and Discussion

### 3.1. Disinfectants inactivation kinetics of *E. coli* in lake water

Inactivation by ferrate and monochloramine following 60 min exposure was mostly dose-dependent over the treatment concentrations of 11.2-55.8 mg/L as Fe for ferrate and 1.7-5.6 mg/L as Cl<sub>2</sub> for monochloramine (Fig. S2). In both cases, there was a plateau in the dose response at approximately 3 to 4-log<sub>10</sub> inactivation of cells at dose ranges of 16.8-33.5 mg/L as Fe for ferrate and 2.5-3.5 mg/L as Cl<sub>2</sub> for monochloramine (Fig. S2). Ferrate and monochloramine exposure resulted in observably different

inactivation kinetics of *E. coli* in amended lake water media (Fig. 1). Application of ferrate at concentrations of 11.2 and 22.3 mg/L as Fe resulted in approximately  $1.0 \pm 0.2$  and  $4.1 \pm 0.2$ -log<sub>10</sub> inactivation of *E. coli*, respectively, within 20 min (Fig. 1A). Though the time required for inactivation was different for the two ferrate concentrations (7 min for 11.2 mg/L as Fe and 20 min for 22.3 mg/L as Fe), the corresponding Chick's law rate constants were not significantly different between the ferrate doses of 11.2 mg/L as Fe ( $0.16 \pm 0.07$  min<sup>-1</sup>) and 22.3 mg/L as Fe ( $0.14 \pm 0.05$  min<sup>-1</sup>) ( $p=0.70$ ) (Fig. S2). This finding is in agreement with previous observations that *E. coli* inactivation kinetics were not dependent on ferrate concentrations (Cho et al., 2006).

Monochloramine treatment at 2.5 mg/L and 4.6 mg/L as Cl<sub>2</sub> resulted in  $3.6 \pm 0.1$  and  $6.3 \pm 0.2$ -log<sub>10</sub> inactivation after 60 min, respectively (Fig. 1B). The calculated Chick's law rate at 4.6 mg/L as Cl<sub>2</sub> ( $0.10 \pm 0.02$  min<sup>-1</sup>) of monochloramine treatment was significantly higher compared to that at 2.5 mg/L as Cl<sub>2</sub> ( $0.05 \pm 0.01$  min<sup>-1</sup>) ( $p=0.02$ ). These rates were lower than those for ferrate, pointing to slower inactivation rates compared to ferrate. Ferrate decay in amended lake water occurred within the first 7 – 15 minutes of exposure (Fig. S3), whereas residual monochloramine concentration persisted beyond 60 min (Fig. S4; See Text S2 for additional discussion on CT values). Additionally, the presence of organic matter (TOC= 56.7 mg/L; Table S2) in the post-growth lake water is an important factor to consider as it could exert an oxidant demand (Deng et al., 2018), which could diminish the disinfectants' availability for disinfection. For example, previous studies have shown that higher organic load in source waters decreased the inactivation potential of ferrate against cyanobacteria (Fan et al., 2018) and MS2 bacteriophage (Wu et al., 2019), due to reactions between ferrate and available organic matter. Additionally, compared to other strong oxidants, ferrate

impacted the electron donating capacity of Suwanee River NOM less than other strong oxidants, suggesting limited production of reactive oxygen species (ROS) during ferrate-organic matter interactions (Goodwill et al., 2016).

The difference in inactivation rates suggests that ferrate exhibited a faster mode of disinfection compared to monochloramine that was concentration independent. Similarly, many other disinfectants (ozone, permanganate, and chlorine dioxide) result in faster cellular inactivation compared to monochloramine (Ramseier et al., 2011). Differences in microbial inactivation kinetics among various chemical disinfectants have been previously noted (Gyürek and Frinch, 1998; Ramseier et al., 2011). For example, an initial lag in inactivation has been attributed to slower diffusion of a disinfectant towards specific cellular targets and/or the need for a disinfectant to target multiple cellular components to inactivate cells (Gyürek and Frinch, 1998; Jacangelo et al., 1991). The multiple-hit model of inactivation has been proposed as the likely scenario for inactivation by halogen-based disinfectants, such as monochloramine (Jacangelo et al., 1991). In this model, the inhibition of multiple biological processes is required before an appreciable level of bacterial inactivation is observed. On the other hand, ferrate is a strong oxidant ( $E^0=2.2V$ , acidic media (Wood, 1958)) compared to monochloramine ( $E^0=1.5V$ , pH 0 (Rajasekharan et al., 2007)) and, therefore, different oxidative reactions may be involved. In addition, ferrate can generate multiple reactive intermediates such as perferryl(V), ferryl(IV), and other ROS such as hydrogen peroxide (Lee et al., 2014). While it is unclear which ferrate-derived species contribute to the inactivation of *E. coli*, ferrate exposure may induce rapid cellular damage through non-selective protein

degradation and injury to the cell membrane (Hu et al., 2012; Ramseier et al., 2011; Sharma et al., 2015). The variations in the observed inactivation kinetics in response to ferrate and monochloramine herein further suggest that the disinfectants' underlying chemistry and reactivity towards different cellular targets are factors that could govern their performance and effectiveness.

### 3.2. *E. coli* viability in response to different disinfectants

The proportion of cells that were viable in cultures treated with monochloramine or ferrate was significantly different ( $p=0.0003$ ). (Fig. 2), despite both treatments achieving similar maximal levels of *E. coli* inactivation ( $\sim 3 \log_{10}$ ) as measured by plate counting (Fig.1). Compared to untreated controls and monochloramine treated cultures, the loss of cellular viability was significantly higher in cultures treated with ferrate (Fig. 2). As cell viability assessment by LIVE/DEAD analysis is based on differences in membrane integrity, these results suggest that ferrate treatment resulted in significant membrane damage whereas monochloramine did not. The chemical-based differences (reactivity and selectivity) between ferrate and monochloramine could explain the significantly higher loss of cellular viability and greater membrane damage in cultures treated with ferrate compared to monochloramine. Ramseier et al. (2011) reported damage kinetic rates of drinking water bacterial cell membranes due to ferrate ( $\sim 9 \times 10^{-3} \text{ L/mg} \times \text{min}$ ), which were two orders of magnitude higher than monochloramine ( $\sim 4 \times 10^{-5} \text{ L/mg} \times \text{min}$ ) treatment. The high reactivity of ferrate towards thiol groups ( $10^5 \text{ M}^{-1} \text{ s}^{-1}$  at pH 8) (Sharma et al., 2011) has been proposed to be one of the factors causing rapid and irreversible degradation of cysteine and methionine peptides in ferrate exposed viruses (Hu et al., 2012; Kazama, 1994). On the contrary, monochloramine could be inducing VBNC state in exposed cells as indicated by the large difference between the plate

count and LIVE/DEAD results. Chlorination and UV irradiation have been shown to induce VBNC among several bacterial species, including *E. coli*, *Pseudomonas* spp., *Aeromonas* spp. and *Salmonella* Typhimurium by triggering growth inhibition and arrest as opposed to compromising cellular components (Chen et al., 2019, 2018; Fan et al., 2018; Guo et al., 2019; Lin et al., 2017; Ye et al., 2020; Zhang et al., 2015). In these studies, evidence of intact cells and reduced metabolism suggested that cells remained viable despite losses in culturable cell concentrations post-treatment. The similarity in the losses in culturability between ferrate and monochloramine treated cultures suggests there could be an overlap in certain cellular responses. However, the discrepancy in cell viability could be driven by unique molecular responses to each disinfectant.

### 3.3. General shifts in global gene expression in response to disinfection

To further understand the molecular responses triggered by each disinfectant, global gene expression profile was analyzed following 15 min of disinfection. Significant differential expression of 931 (22% of all *E. coli* genes) and 230 (5% of all *E. coli* genes) genes were observed upon monochloramine and ferrate exposure, respectively (Fig. 3; Fig. S6). In comparison, a total of 29 (0.68% of all genes) genes were differentially expressed in control cultures (Fig. 3). There were 10 times as many genes upregulated due to monochloramine compared to ferrate treatment. A greater proportion of *E. coli* cells treated with ferrate were inactivated by 15 min (Fig. 1) and determined to be unviable by 60 min compared to monochloramine treatment (Fig. 2). Therefore, the differences in cell inactivation and viability could have driven the observed disparity in gene expression profiles. Nonetheless, both ferrate and monochloramine treated



cultures shared a subset of differentially expressed genes (210 genes), of which 93% were downregulated, suggesting the certain pathways of cellular inactivation may be similar (Fig. 3, Table S3).

### 3.4. Downregulation of a similar subset of genes by disinfectants

Both monochloramine and ferrate treatments led to the downregulation of genes related to translation and transcription regulation, RNA and protein metabolism, and cytokinesis (Fig. 4). For example, both ferrate and monochloramine treatment led to the downregulation of a similar subset of genes related to the 30S ribosomal subunit (*rpsKLO*) and tRNA synthetases (*alaS*, *asnS*, *hisS*, *thrS*) by at least 2.1  $\log_2$  (Table S3). Main global transcription regulators such as *crp*, *fur*, *ada*, and *slyA*, were also downregulated by at least 2.7 and 2.8- $\log_2$  following ferrate and monochloramine disinfection, respectively (Table S3). Both disinfectants downregulated genes related to cytokinesis processes (e.g. *zapA*, *nlpD*, *damX*, and *ftsQ*) by at least 2.4  $\log_2$  (Table S3). These genes are responsible for cell septum formation during reproduction; loss of their gene products could signal stress in cell replication (Asakura and Kobayashi, 2009).

Ribosomal proteins, amino acid transporters, nucleotide synthesis, and lipoprotein metabolism in *E. coli* were also downregulated following exposure to sub-lethal concentrations of chlorine (Wang et al., 2009), hydrogen peroxide (Chang et al., 2002; Wang et al., 2009) and monochloramine (Berry et al., 2010), which is in agreement with findings in this study. Downregulation of genes involved in translation and transcription machinery could indirectly disrupt downstream biological functions important for protein synthesis and metabolism (Chang et al., 2002). Additionally, widespread reduction in

the expression of ribosomal-associated proteins has been associated with reduced metabolism and VBNC induction in response to environmental stressors (Asakura et al., 2007; Ramamurthy et al., 2014). The broader range of downregulated genes involved in gene expression under monochloramine treatment (approximately 130 genes) compared to ferrate (45 genes) suggests that apparent inactivation by monochloramine could be the result of reduced metabolism and VBNC induction rather than cellular damage.

### **3.5. Significant downregulation of stress response genes under monochloramine treatment**

Monochloramine treatment led to the significant downregulation of up to 109 genes involved in general stress responses such as *dps*, *rpoS*, and *uspA* by 2.7, 3.7 and 3.0- $\log_2$ , respectively. Genes related to acid resistance (*hdeAB*), heat and osmotic stress (*otsAB*), and UV irradiation/DNA damage (*dinQ*, and *uspE*) were downregulated by at least 4.0, 2.9, and 2.5- $\log_2$ , respectively (Table S3). This is reflected by the significant over-representation of the 'response to stress' GO term among genes downregulated by monochloramine (1.4;  $p=0.01$ ) (Fig. 4). In this work, cultures were treated at late stationary phase and under oligotrophic growth conditions, which typically trigger cellular responses against starvation and other stressors (Navarro Llorens et al., 2010). It is possible the additional stress of disinfection could have contributed to the shut-down of *E. coli*'s induced state of general stress response.

To illustrate the shift in the mRNA abundance of genes due to disinfection, the distribution of expressed genes was plotted across normalized transcript count data pre and post-disinfection (Fig. S7). Prior to disinfection, genes spanned a wide distribution of normalized counts irrespective of GO category. For example, functional categories

such as the response to stress, starvation, and osmotic stress as well as cellular homeostasis had similar, broad distributions of genes ( $10^1$ - $10^5$  counts) while the median ranged between 500 - 5000 transcript counts (Fig. S7A). Following disinfectant treatment, a narrower distribution of genes ( $10^2$ - $10^4$  counts) was observed for both monochloramine and ferrate treatments. The median shifted to a narrower range of 900-1000 counts across all GO categories analyzed after 15 min of exposure to the disinfectants (Fig. S7B, C). Key genes induced during stationary phase (*bola*, *yeaH* and *rpoS*) (Navarro Llorens et al., 2010) had high transcript counts pre-disinfection (30,000 – 700,000), but were found to be significantly downregulated following monochloramine treatment (Table S3).

A similar transcriptomic profile of *E. coli* in response to monochloramine exposure was also observed by Berry et al. (2010) in which cells were grown under nutrient-limited conditions. It has been suggested that the exposure of starved cells to disinfectants may have triggered a transcriptomic response similar to bacterial cells exhibiting stringent response during growth arrest (Berry et al., 2010; Chang et al., 2002). Contrary to our findings, Wang et al. (2010) showed that pathogenic *E. coli* O157:H7 strains cultivated under rich growth conditions responded to chlorine treatment by upregulating universal stress response gene such as *uspA*, *dps* and *rpoS*. The discrepancy in findings is attributed to the different cultivation (rich versus nutrient-limited) methods that could impact the cells' expression of growth-associated stress response genes (Tao et al., 1999). For example, the expression levels of universal stress response regulators (e.g. *rpoS* and *uspA*) and stress-related genes are typically induced and higher in stationary phase or in cells growing in nutrient-limited environments compared to cells growing in favorable and nutrient-rich conditions (Chang et al., 2002; Navarro Llorens et al., 2010;

Nyström, 2004). Growth conditions pre-disinfection were shown to significantly alter the susceptibility of bacterial cells to disinfectants. Lisle et al. (1998) showed that adaptation to starvation enhanced tolerance of *E. coli* to chlorination, which points to the significance of growth conditions and associated stress responses on *E. coli* susceptibility and physiological responses post-disinfection.

### 3.6. Gene upregulation under monochloramine treatment

Genes significantly upregulated by monochloramine exposure were related to four main biological processes that involved biological adhesion, localization, metabolism, and cellular organization (Table 1). The expression of genes related to the formation of fimbrium structures, pilus organization, and cellular adhesion (*fimCFI*, *ygiL*, *yfcQR*, *yehA*, *ydeQRS*, *elfA*, *sfmAF*, *yadVN*) increased after monochloramine exposure (Table S3). Similarly, studies on *E. coli* (Holder et al., 2013) and *Salmonella enterica* (Wang et al., 2010) exposed to monochloramine and chlorine identified an increase in the expression of genes related to fimbriae and pili formations known to enhance adhesion and biofilm formation. Formation of biofilms has been generally associated with resistance of pathogens against biocides (Berry and Xi, 2009; Tachikawa et al., 2005), suggesting that the *E. coli* cells were exhibiting survival mechanisms in response to monochloramine exposure.

Localization, specifically carbohydrate and organic substance transport, was significantly enriched following monochloramine treatment (Table 1). Some genes encoding proteins involved in the phosphoenolpyruvate carbohydrate phosphotransferase system (PTS) (*agaBCS*, *frwABCD*, *fryA*, *cmtAB*) were upregulated following chloramines exposure (Table S3). PTS is the major carbohydrate transport system in *E. coli* involved in catalyzing the phosphorylation of carbohydrates during

membrane translocation (Kotrba et al., 2001). The upregulation of PTS genes suggests that oxidative stress triggered by monochloramine treatment may stimulate carbohydrate movement across the membrane in an already nutrient-deprived environment. Similarly, paraquat exposure in *E. coli* has been shown to induce the expression of genes encoding glucose transport machinery (Rungrassamee et al., 2008). In contrast, a genome wide transcriptomic analysis of two different strains of *S. enterica* (pre-grown in rich growth media) to chlorine stress showed that both strains downregulated PTS related genes and carbohydrate metabolism (Wang et al., 2010). However, cellular responses to oxidants can vary depending on the organism as well as growth conditions such as nutrient content of the growth media used (Berry and Xi, 2009; Oladeinde et al., 2018; Small et al., 2007). This may explain the discrepancy as well as highlight important factors that can cause deviations in bacterial responses to the same disinfectants.

25 genes related to metabolism involved in the breakdown of non-glucose compounds were upregulated by monochloramine exposure (Table 1). Those metabolism-related genes mainly encoded proteins associated with the breakdown of small organic molecules such as rhamnose (*rhaABDM*) and other organonitrogen compounds such as threonine (*tdcAFD*) and ethanolamine (*eutGPNQ*) (Table S3). Modulating carbon flow and metabolism has been observed among *E. coli* as a defense mechanism against oxidative stress (Rungrassamee et al., 2008). It is generally hypothesized that the activity of carbon metabolism is stimulated to regenerate important reduced cofactors (e.g., NADH, NADPH) that may become depleted as a result of oxidative stress (Holder et al., 2013). In this present study, the combination of oligotrophic conditions in the growth media, late stationary phase conditions of the cells, and the introduction of an

additional stress through disinfection may have triggered the activation of non-glucose carbon utilization pathways to enhance cell survival.

### 3.7. Gene upregulation under ferrate treatment

Ferrate-treated *E. coli* cultures exhibited limited gene upregulation (27 genes) compared to downregulation (203 genes) (Fig. 3). Although not significant ( $p > 0.1$ ), upregulation by ferrate exposure appeared to enrich cell redox homeostasis and short-chain fatty acid metabolism (Tables 1, S3). Two genes that were significantly upregulated by ferrate were *grxA* and *trxC*. Both genes encode cytoplasmic disulfide reducing proteins (thioredoxins and glutaredoxins) that help maintain the reduced state of cells (Carmel-Harel and Storz, 2000). These genes are part of the OxyR regulon, which is important for alleviating oxidative stress (Prieto-Álamo et al., 2000). Induction of oxidative stress related genes in response to elevated levels of ROS has been well established, in which transcription factors such as OxyR and SoxR are activated in the presence of oxidants at high concentrations (Dukan and Touati, 1996; Gundlach and Winter, 2014; Seo et al., 2015).

The limited diversity of upregulated genes and enriched biological functions under ferrate treatment compared to monochloramine suggests that bacterial transcriptomic responses, despite some similarities, remain unique to different disinfectants (Small et al., 2007).

### 3.8. Time-dependent upregulation of oxidative stress genes post-disinfection

Ferrate treatment led to the significant upregulation of *trxC* and by  $24.0 \pm 6.6$  and  $12.8 \pm 4.3$  folds, respectively, within 5 min of exposure compared to pre-disinfection as determined through qRT-PCR analysis ( $p < 0.05$ ; Fig. 5A). The relative expression of *trxC* and *grxA* in response to ferrate disinfection decreased over the 60 min exposure period. The expression of *soxS*, *recA*, *rpoS*, and *gshB* was not significantly affected by exposure to ferrate (Fig. 5A). Monochloramine treatment resulted in a significant upregulation of *trxC*, *grxA*, and *soxS* by  $27.6 \pm 4.9$ ,  $57.4 \pm 6.4$ , and  $9.0 \pm 0.4$  folds, respectively, within 5 min of exposure (CT=9 mgxmin/L) ( $p < 0.05$ ; Fig. 5B), followed by an increase in relative expression levels by 30 min (CT=47 mgxmin/L). Monochloramine treatment also led to the significant upregulation of *rpoS* ( $4.9 \pm 0.2$  folds), *recA* ( $5.2 \pm 0.2$  folds), and *gshB* ( $7.8 \pm 0.3$  folds) at 30 min of treatment ( $p < 0.05$ ; Fig. 5B).

The upregulation of OxyR regulated genes (*trxC* and *grxA*) within 5 min of exposure to ferrate or monochloramine treatment suggests a rapid OxyR-dependent response to either disinfectant. Upregulation of *soxS* (SoxR-regulated gene) in response to monochloramine, but not ferrate, indicates that although both disinfectants triggered an OxyR-dependent oxidative stress response, only monochloramine triggered a SoxR-dependent response. The observed differences in *soxS* upregulation could be due to differences in the extent of SoxR activation by the disinfectants. While OxyR could remain in the active and oxidized form following removal of oxidative stress (Åslund et al., 1999), SoxR has been shown to revert to the inactive reduced form within few

minutes of oxidative stress withdrawal (Ding and Demple, 1997). Rapid ferrate decay may have prompted rapid reversal of the SoxR oxidized state, whereas slower monochloramine decay could have sustained oxidative stress levels, which maintained the active states of OxyR and SoxR. The upregulation of genes related to general stress response such as *recA* (DNA repair), *rpoS* (general stress response), and *gshB* (glutathione biosynthesis) under monochloramine treatment, but not ferrate, further suggests that the two disinfectants trigger different adaptive responses to oxidative stress (Small et al., 2007).

The expression levels of the upregulated genes by ferrate and monochloramine treatment also varied with exposure time (Fig. 5). For example, upregulation of the OxyR-dependent gene, *grxA*, peaked at 5 min and 30 min following ferrate and monochloramine treatment, respectively. Time course variation in gene expression has been observed for OxyR dependent genes following *E. coli* exposure to hydrogen peroxide (Micha et al., 1999) and paraquat (Prieto-Álamo et al., 2000). Upregulation of other stress related genes (*rpoS*, *gshB*, *recA*) following monochloramine treatment occurred after 30 min of exposure, suggesting that certain cellular adaptive responses could increase with exposure time (Holder et al., 2013). The time-dependent variations in relative gene expression in response to disinfectants emphasize that gene expression driving certain biological functions, such as oxidative stress responses, are dynamic and time-sensitive, and are driven by variations in the inactivation kinetics of the different disinfectants.

#### 4. Conclusions

Deeper understanding and innovation in disinfection processes will become more important as potable water reuse gain acceptance, especially in water scarce regions.



Understanding the mode of action of disinfectants is especially critical since it could inform the selection of appropriate disinfectant and dosage level needed to prevent pathogen regrowth and resistance development. In this study, complex molecular and growth-related responses were found to be involved in the inactivation of *E. coli* by two chemically different disinfectants. The following conclusions were found:

- Chemically different disinfectants (ferrate vs. monochloramine), resulted in a similar loss in culturable cell concentrations; however, ferrate disinfection was faster and resulted in significantly greater reduction of cell viability compared to monochloramine.
- Contrary to ferrate treatment, monochloramine treatment resulted in the widespread downregulation of genes related to translation and transcription, which may have contributed to reduced metabolism and VBNC induction in *E. coli*.
- Downregulation of stress-related genes under monochloramine disinfection was attributed to the shutdown of existing late stationary phase stress responses.
- VBNC induction in response to monochloramine treatment challenges drinking water treatment and monitoring since VBNC pathogenic bacteria are undetectable using traditional culture-based methods and retain virulence. Hence, evidence of VBNC induction in response to monochloramine disinfection but not to ferrate calls for research efforts to further investigate alternative and more effective disinfectants compared to monochloramine.
- The influence of growth-phase related transcriptomic profile on bacterial responses suggests that more work is needed to better understand how source water growth conditions could impact pathogen susceptibility to disinfectants.

**Conflicts of interest**

The authors declare that there are no known competing financial interests or personal relationships that could have influenced the work and findings of this paper.

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**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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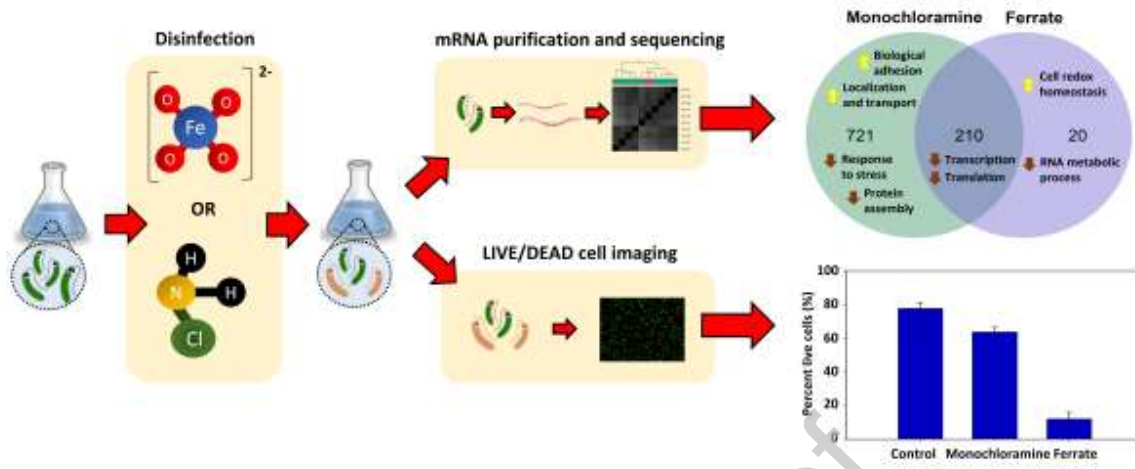
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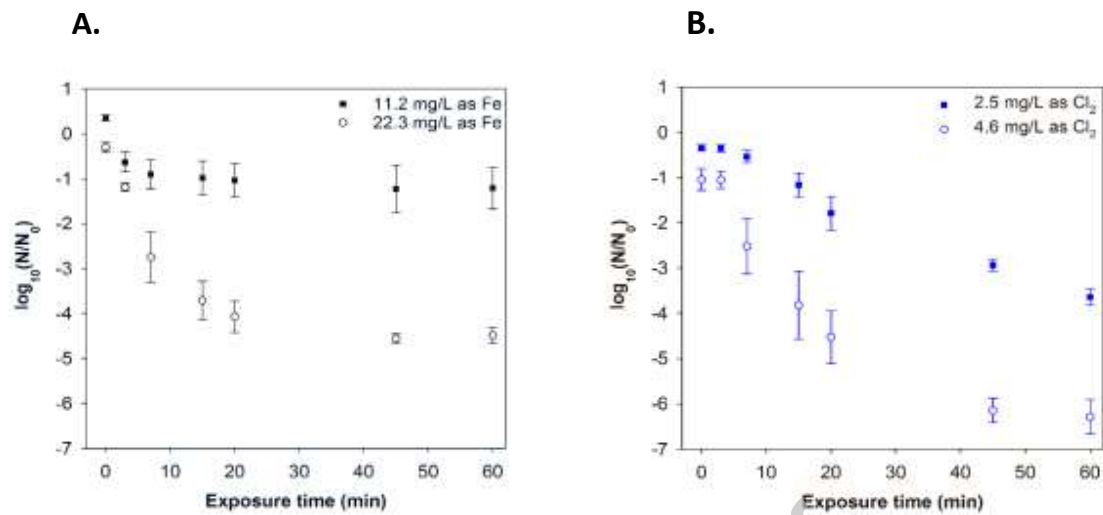
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**Table 1. Enriched biological functions as a result of the upregulation of genes by either ferrate or monochloramine.** Parent categories are provided to allow for categorization of the child GO classes.

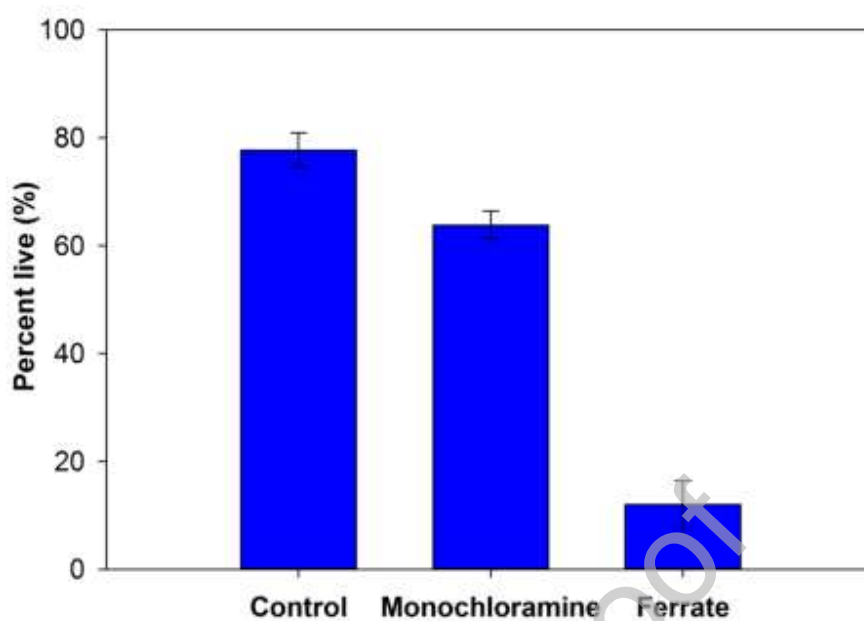
Functional Terms	Number of differentially expressed genes	Fold enrichment	Benjamini-Hochberg ( <i>p</i> -value)
<b>Ferrate treatment</b>			
<b>Cellular process</b>			
Cell redox homeostasis	2	20.5	0.97
<b>Metabolic process</b>			
Short-chain fatty acid metabolic process	9	2.5	0.53
<b>Monochloramine treatment</b>			
<b>Cellular component organization and biogenesis</b>			
Pilus assembly	6	8.5	0.059
<b>Localization</b>			
Carbohydrate transport	16	2.7	0.072
PEP-dependent sugar phosphotransferase system	9	3.0	0.48
<b>Metabolic process</b>			
Fatty acid metabolic process	9	2.5	0.53
Short-chain fatty acid metabolic process	7	7.2	0.073
Alcohol catabolic process	6	3.1	0.67
Organic acid catabolic process	15	1.6	0.74
Amine catabolic process	5	3	0.8
<b>Cellular metabolic process</b>			
DNA recombination	11	2	0.72
<b>Control</b>			
<b>Metabolic process</b>			
Fatty acid metabolic process	3	37.6	0.17

## Graphical abstract

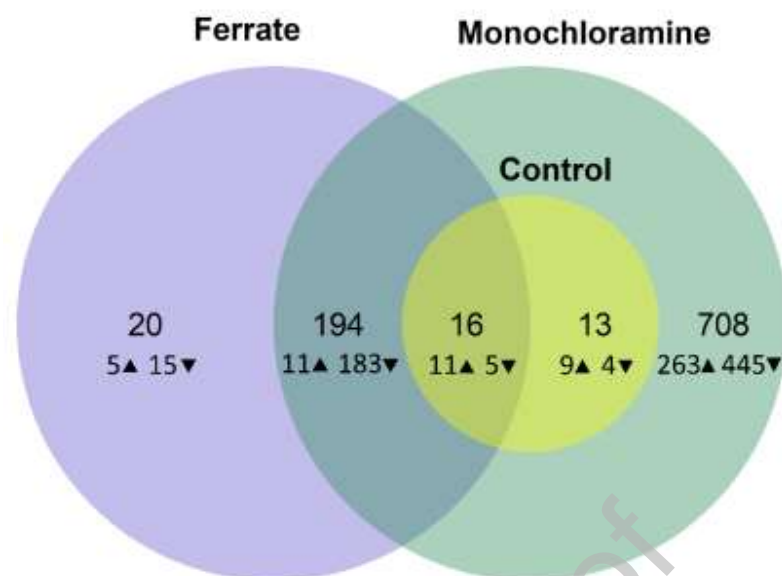




**Fig.1 - Kinetics of *E. coli* inactivation in response to different disinfectants.**  $\text{Log}_{10}$  inactivation over time following exposure to (A) 11.2 and 22.3 mg/L as Fe of ferrate and (B) 2.5 and 4.6 mg/L as  $\text{Cl}_2$  of monochloramine. Error bars represent one standard error of at least triplicate samples. Initial cell concentration ( $N_0$ )  $\sim 10^8$  CFU/mL; pH = 8.4; room temperature.

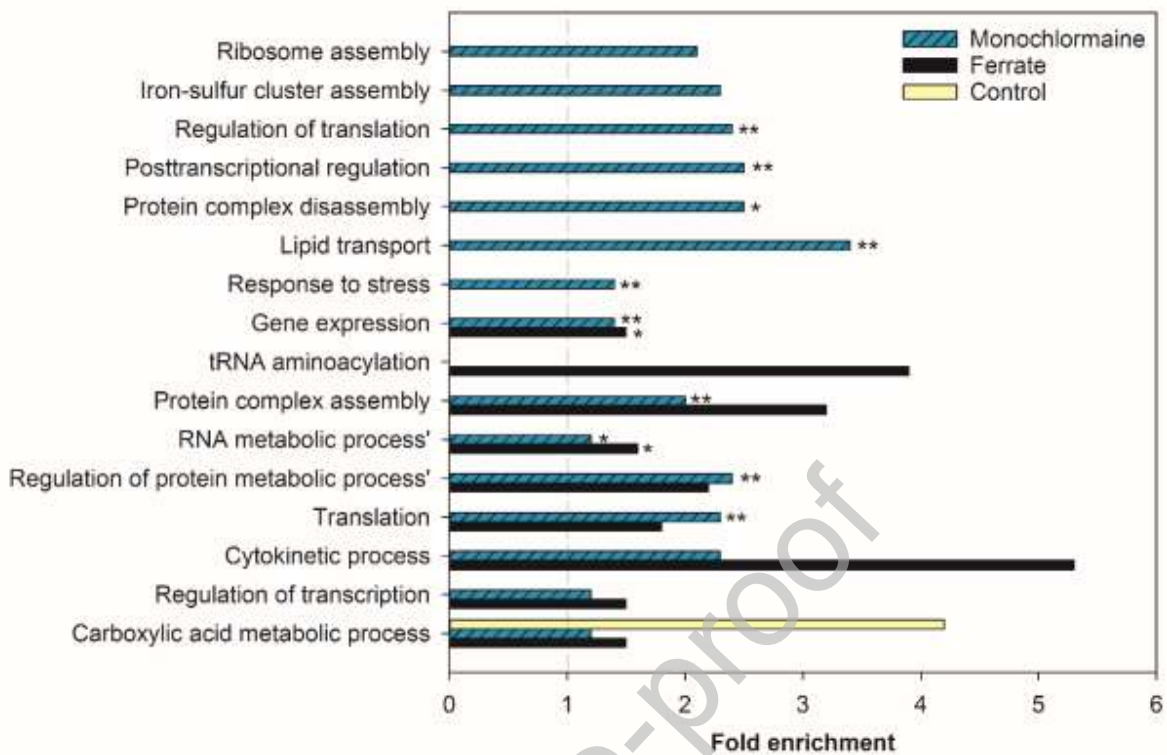


**Fig. 2 - Percentage of cells that were viable after 60 min of treatment with 11.2 mg/L as Fe of ferrate or 2.5 mg/L as Cl<sub>2</sub> of monochloramine or no treatment (control). Error bars represent one standard error of at least triplicate samples. Initial cell concentration (N<sub>0</sub>) ~10<sup>8</sup> CFU/mL; pH= 8.4; room temperature.**



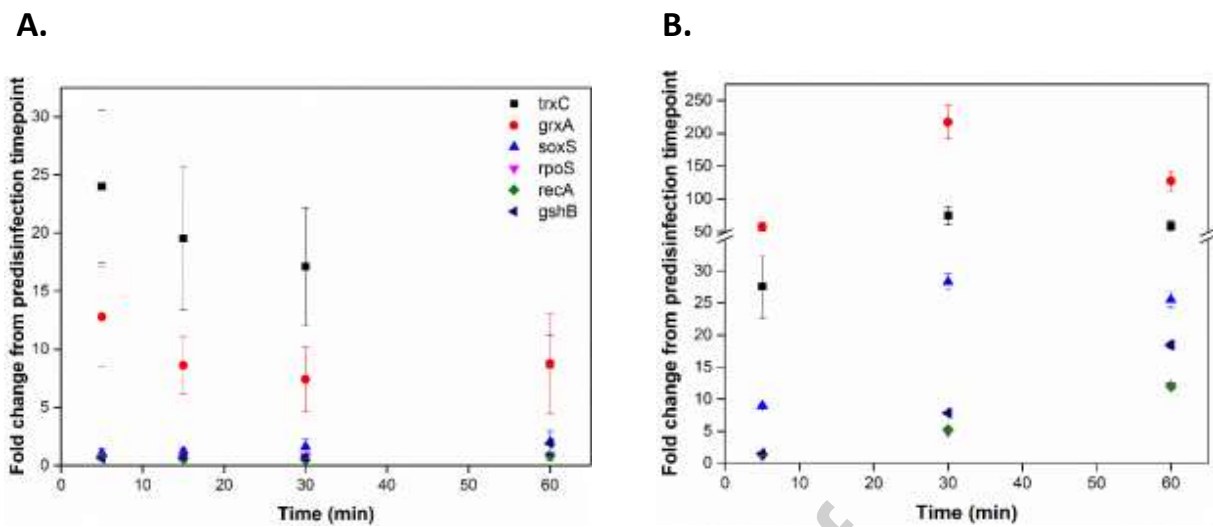
**Fig. 3-** Venn diagram illustrating the distribution of genes following a 15 min exposure to 22.3 mg/L as Fe of ferrate, 2.5 mg/L as Cl<sub>2</sub> of monochloramine or water (control). Direction of arrows indicate the direction of regulation and numbers indicate numbers of genes. Arrow direction: up (upregulation) and down (downregulation).





**Fig. 4- Biological functions (GO Terms) that are enriched among the subset of downregulated genes in *E. coli* following exposure to monochloramine or ferrate.**

Genes representing those functions were differentially expressed (downregulated) under either ferrate or monochloramine treatment. Dashed line represents fold enrichment cut-off with values above 1.0 considered enriched. Single (\*) and double asterisks (\*\*) denote GO terms significant at  $p < 0.1$  and  $p < 0.05$  (Hochberg-Benjamini), respectively. GO terms with no asterisks were not significant ( $p > 0.1$ ). Absence of bars indicate that the GO term was not enriched in the respective data set.



**Fig. 5-** Fold change in gene expression over time relative to the pre-disinfection time point ( $t=0$ ) using qRT-PCR in response to (A) 22.3 mg/L as Fe of ferrate and (B) 22.3 mg/L as  $\text{Cl}_2$  of monochloramine. Fold change values above 1.0 were considered upregulated. Error bars correspond to one standard error of three biological replicates.