**Individual and combined use of ultraviolet (UV) light with hydrogen
peroxide, peracetic acid, and ferrate(VI) for wastewater disinfection**

by

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Abstract

This work explores the use of chlorine-free chemicals hydrogen peroxide (HP), peracetic acid (PAA), and ferrate(VI) as well as physical disinfection method, ultraviolet light (UV), alone and combination for wastewater disinfection. To evaluate treatments the culturability of indicator-organism *Escherichia coli* was compared to that of spore-forming *Bacillus subtilis* and *C. perfringens*. Remaining culturable and viable *E. coli* were studied using a method combining propidium monoazide (PMA) and quantitative polymerase chain reaction (qPCR). Changes to culturability were monitored for up to 48 hours after treatment to evaluate regrowth, reactivation and resuscitation levels.

In **Chapter 4** culturable and viable *E. coli* were studied following disinfection with sodium hypochlorite (NaClO), HP, PAA, ferrate(VI), and ultraviolet light (UV). All methods reduced culturable *E. coli* to below 2 log CFU/mL but 3-6 log CCE/mL of *E. coli* remained viable following chemical treatments. The largest reduction of viable cells was achieved with Ferrate(VI) followed by PAA, NaClO, and HP.

In **Chapters 5 and 6** the use of UV and ferrate(VI) both alone and in combination was studied. The combination of 40 mJ/cm² UV and 9 mg/L ferrate(VI) provided an additional 80% reduction in coliform bacteria compared to the use of UV alone. The use of ferrate(VI) increased turbidity by up to 95.6%. UV fluence of 40 mJ/cm² reduced *C. perfringens* 95.5% but neither ferrate(VI) nor combining UV and ferrate(VI) offered further reductions. A concentration of 9mg/L of ferrate(VI) reduced viable *E. coli* by 0.9

log CCE/mL and by 2 logs CCE/mL when combined with 40 mJ/cm². No regrowth following ferrate(VI) was observed, however regrowth and reactivation were observed after 40 mJ/cm² UV as well as following combined treatment.

In **Chapter 7** the use of UV and PAA were studied. Combining 40 mJ/cm² UV and 2.3 mg/L PAA allowed for an additional 1.7 log reduction in culturable *E. coli* but no additional reduction in *B. subtilis* was observed when compared to the use of UV alone. The combined treatment offered an additional 0.8 log reduction in viable *E. coli*. Regrowth was observed after a lag period between 6 and 24 hours without additional nutrient supplementation.

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"Morning, boys. How's the water?"

– David Foster Wallace, *This is Water: Some Thoughts, Delivered
on a Significant Occasion, about Living a Compassionate Life*

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Nomenclature

AA	Acetic acid		
ANOVA	Analysis of variance	NTU	Nephelometric turbidity units
AODC	Acridine orange direct count	ORP	Oxidation-reduction potential
BOD	Biochemical oxygen demand	PAA	Peracetic acid
BDOC	Biodegradable dissolved organic carbon	PAB	Particle associated bacteria
bp	Base pairs	PBS	Phosphate buffered solution
<i>B.subtilis</i>	<i>Bacillus subtilis</i>	PCR	Polymerase chain reaction
CAWQ	Canadian Association on Water Quality	PMA	Propidium monoazide
CCE	Calculated CFU equivalents	qPCR	Quantitative polymerase chain reaction
CFU	Colony forming units	RNA	Ribonucleic acid
COD	Chemical oxygen demand	RT-PCR	Reverse transcriptase polymerase chain reaction
Ct	(concentration) x (time)	SS	Suspended solids
DBP	Disinfection by-products	TDS	Total dissolved solids
DNA	Deoxyribonucleic acid	THM	Trihalo methane
dNTP	Deoxyribonucleotide triphosphate	TOC	Total organic carbon
DPD	N,N-diethyl-p-phenylenediamine sulphate	TS	Total solids
DOC	Dissolved organic carbon	TSS	Total suspended solids
DSMO	Dimethyl sulfoxide	US EPA	United States Environmental Protection Agency
DVC	Direct viable count	UV	Ultraviolet irradiation
<i>E. coli</i>	<i>Escherichia coli</i>	Tm	Annealing temperature
HAA	Halo acetic acids	TSA	Tryptic soy agar
HP	Hydrogen Peroxide	VBNC	Viable but non-culturable
LP-UV	Low-pressure ultraviolet radiation	WEAO	Water Environment Association of Ontario
MP	Medium-pressure	WEF	Water Environment Federation
MP-UV	Medium-pressure ultraviolet radiation		

Chapter 1

1 Introduction

Disinfection is often the final wastewater treatment process and is our last line of defense before wastewater is released into the environment. Its purpose is to reduce the concentration of enteric pathogenic microorganisms and reduce the risk to the public and the environment. Compliance testing at wastewater treatment plants (WWTP) relies on monitoring select indicator organisms at the point before wastewater is discharged.

Chlorine, the most commonly used disinfectant, works by oxidizing cellular components leading to cell death. It is inexpensive and effective at reducing concentrations of culturable indicator organisms such as *Eschericia coli* and fecal coliforms; however, in the presence of residual organic material, which is present at high concentrations in wastewater, chlorine can lead to the formation of disinfection by-products (DBPs). DBPs such as trihalomethanes (THMs) and halo acetic acids (HAAs) are harmful to human health and can be toxic to aquatic ecosystems (Adin et al., 1991; Fawell et al., 1997). For this reason, recent the Canadian *Wastewater Systems Effluent Regulations*, now requires treatment plants across the country to limit the release of chlorine into Canadian waterways to concentrations of less than or equal to 0.02 mg/L (Government of Canada, 2012). Many WWTPs are dealing with the regulation by quenching chlorinated wastewater effluents with sodium sulfates. However, this solution requires

additional treatment which can add undesirable additional costs to a WWTP. In addition, DBPs can still be formed in chlorine contact chambers. There is a need to explore alternative chlorine-free disinfection technologies for wastewater disinfection that both limit chlorine residuals and further reduce the potential formation of DBPs.

Alternatives to chlorine include physical methods, such as ultraviolet light (UV), as well as chlorine-free chemicals, which may include hydrogen peroxide (HP), peracetic acid (PAA), and ferrate(VI). UV disinfects by changing the nucleic material of cells that render cells incapable of replication and causing diseases. However, particles in wastewater may prevent or shield UV light from reaching microorganisms in water, and limit the effectiveness of the treatment. Following exposure to UV, partially inactivated cells or cells in an injured state, may repair damaged nucleic material and regain the ability to replicate (Knudson, 1986; Oguma et al., 2002; Quek and Hu, 2008). While UV is governed by light intensity and time of exposure, concentration and contact time govern chemical disinfection. The alternative chlorine-free chemicals listed above are, similarly to chlorine-compounds, oxidants but reduced risk of forming halogenated DBPs because they are chlorine-free (Dell'Erba et al., 2007; Monarca et al., 2002a; Sharma, 2002; Tchobanoglous et al., 2003). Additionally, recent studies have found that the use of two disinfectants in combination may have synergistic effects (Cho et al., 2006a; Rennecker et al., 2000).

The intent of this study was to investigate the use of UV and chlorine-free chemicals both alone and in combination for wastewater disinfection. The primary goal of this

research was to make a contribution to the field of wastewater disinfection by investigating the response of different bacteria after treatment as well as compare the culturable and viable but nonculturable (VBNC) state of bacteria. The research approach drew upon the fields of environmental engineering, microbiology, and molecular biology to incorporate the use of cutting edge tools and techniques to understand the effects of each treatment better. Table 1.1 summarizes how the tools and techniques from each subject area were incorporated into the study. This research combined culture and molecular methods. Indicator organisms such as *Escherichia coli*, fecal coliforms, and total coliforms are widely used in compliance testing at WWTPs; however, do not provide any information about the survival of more robust bacteria. For this reason, the culturability of indicator *E. coli* was compared to that of spore-forming bacteria *Bacillus subtilis* and *Clostridium perfringens*. Microorganisms' regrowth potential was monitored with culture methods following treatment.

Table 1.1: Field of study and corresponding tools and techniques employed during the study

Field	Tools and techniques used in the study
Environmental Engineering	<p>Performing controlled bench-scale batch disinfection experiments using secondary wastewater</p> <p>Monitoring indicator organisms and wastewater characteristics using standard methods</p> <p>Examination of chemical and physical disinfection methods</p>
Microbiology	<p>Culturing and maintenance of pure laboratory strains of <i>E. coli</i> and <i>B. subtilis</i></p> <p>Identification of wastewater-indigenous communities of <i>C. perfringens</i>, <i>P. aeruginosa</i></p> <p>Enrichment and isolation of <i>P. aeruginosa</i> colonies from wastewater</p> <p>Colony identification via morphology and cell characteristics</p> <p>Staining and examination of bacteria using molecular stains and fluorescent microscopy</p>
Molecular Biology	<p>Utilizing molecular methods such as DNA isolation, qPCR, PMA-qPCR, and a live/dead viability stain</p> <p>Sequencing and identification of <i>P. aeruginosa</i> from wastewater</p>

Additionally, oxidative stress due to chemical disinfection has been shown to induce bacteria into a VBNC state (Noor et al., 2009; Oliver et al., 2005; Servais et al., 2009) where bacteria remain metabolic active but are no longer able to grow on culture media (Cagua et al., 2014; Oliver, 2005; Oliver et al., 2005; Xu et al., 1982). For this reason, culturability and VBNC *E. coli* were compared. To study VBNC bacteria, researchers have previously used direct viable count (DVC), live/dead viability staining, and reverse transcriptase-polymerase chain reaction (RT-PCR) (Oliver, 2010). More recently a

molecular method combining the use of DNA-intercalating dye propidium monoazide (PMA) and quantitative polymerase chain reaction (qPCR) has been used to investigate dilute and complex matrices such as drinking water (Zhang et al., 2015), wastewater (Bae and Wuertz, 2009; Varma et al., 2009), and sludge samples (van Frankenhuyzen et al., 2013, 2011), and was validated by our research laboratory (Kibbee et al., 2013; Kibbee and Örmeci, 2017). The live/dead viability stain was also used to provide visualization of viable cells before and after treatments.

1.1 Thesis Outline and Hypotheses

This study has been presented in a total of 8 chapters. The current chapter, **Chapter 1**, includes an introduction to the research topic, a description of the research approach as well as a list of hypotheses to be investigated.

Chapter 2 provides a summary of the current state of the literature required to understand the subject of the dissertation in greater detail. This chapter has been prepared for thesis purposes only.

Chapter 3 presents the details of the materials and methods including method validation and laboratory protocols applied throughout the study.

Chapters 4-7 are the primary research chapters of the dissertation and have been prepared as standalone journal articles.

Chapter 4 compares traditional culture methods to that of PMA-qPCR following selected disinfection methods including sodium hypochlorite (NaOCl), HP, PAA, ferrate(VI), and

UV. The PMA-qPCR method was designed to investigate viable *E. coli* in a sample. This research was presented at the National Conference on Water and Wastewater in Whistler, BC in October of 2015 and was awarded the Philip H. Jones Award for best student presentation entitled *“Effectiveness of Chlorine and Chlorine-free alternatives for the Disinfection of Wastewater.”* This chapter has been prepared for the thesis and publication. The hypotheses for this chapter are as follows:

- PMA-qPCR can be used to detect bacteria in the VBNC state following disinfection with sodium hypochlorite, hydrogen peroxide, peracetic acid, ferrate(VI), and UV light.
- Bacteria in the VBNC state are present in wastewater following disinfection with sodium hypochlorite, hydrogen peroxide, peracetic acid, ferrate(VI), and UV light.
- Following disinfection, the quantity of viable *E. coli* is greater than what can be counted culture methods.
- Certain disinfectants are more effective than other at reducing VBNC-bacteria.

Chapter 5 investigated the sequential use of UV and ferrate(VI) for the disinfection of secondary wastewater as an alternative to the application of UV or ferrate(VI) alone. The study compared the culturability of coliform bacteria following each treatment and investigated the effects of turbidity, particle size distribution, and other wastewater characteristics. This chapter has been prepared for the thesis and publication. The hypotheses for this chapter are as follows:

- Ferrate(VI) placed before UV will significantly change the time required for UV
- Ferrate(VI) influences the size and quantity of particles in wastewater.

- Sequential disinfection will allow for increased disinfection capacity when compared to the use of UV alone.
- The use of ferrate(VI) will improve wastewater characteristics such as phosphorous and nitrogen levels.

Chapter 6 presents a microbial analysis of sequential disinfection of UV and ferrate(VI) and includes inactivation of *E.coli*, and *C. perfringens* as well as an evaluation of VBNC *E. coli* after UV and ferrate(VI) when used alone and in sequence. Regrowth of coliform bacteria was also monitored after treatment using culture methods. Chapters 5 and 6 have been presented at the Eastern Canadian Association on Water Quality (CAWQ) conference held in Montreal, QC, in the fall of 2014 and have been written as a blog post for The Canadian Health Adaptations, Innovations, and Mobilization Centre (CHAIM) (<https://carleton.ca/chaimcentre>). This chapter has been prepared for the thesis and publication. The hypotheses for this chapter are as follows:

- Bacteria in the VBNC state are found in wastewater disinfected with UV or ferrate(VI)
- The sequential use of UV and ferrate(VI) allows for an additional reduction of cultured bacteria.
- The sequential use of UV and ferrate(VI) will further reduce the quantity of VBNC *E. coli*
- No regrowth will be observed following disinfection with ferrate(VI)
- No regrowth will be observed following disinfection with UV and ferrate(VI)

Chapter 7 studies the effectiveness of the combined use of UV and PAA for wastewater disinfection and includes a comparison of culturable and VBNC *E. coli*, changes to

culturability of *B. subtilis*, as well as the contribution of HP to the effectiveness of PAA. This chapter has been presented at the 2016 National Water and Wastewater Conference held in Toronto, ON, in November 2016 as well as the Water Environment Association of Ontario (WEAO) Technical Conference held in Ottawa in the winter of 2017. It was also published as a technical paper as part of WEAO's conference proceedings. This chapter has been prepared for the thesis and publication. The hypotheses for this chapter are as follows:

- The combined use of UV and PAA will further decrease culturable bacteria compared to the use of UV alone.
- The sequential use of UV and PAA will further reduce the quantity of VBNC *E. coli*
- No regrowth will be observed following disinfection with PAA
- No regrowth will be observed following disinfection with UV and PAA

Chapter 8 concludes the dissertation by summarizing research outcomes and provides suggestions for future work. This chapter has been prepared for thesis purposes only.

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Chapter 2

2 Background and Literature Review

Since the 1920s, chlorine has been one of the most widely used disinfectants for wastewater worldwide despite its potential to form harmful DBPs and that chlorine residuals have been shown to have toxicological effects (Bolton and Cotton, 2008; Liviak et al., 2010; Plewa et al., 2012; Watson et al., 2012). As an alternative, UV is a physical disinfection method that leaves no chemical residual and has been shown to be effective against chlorine-resistant protozoa such as *Cryptosporidium parvum* oocysts and *Giardia lamblia* (Bolton et al., 1998; Bukhari et al., 1999; Craik et al., 2001; Liberti et al., 2003; Linden et al., 2001). The first commercial UV facility was established in 1910; however, it wasn't until 1955 that the technology was stable enough to be used more routinely (Bolton and Cotton, 2008; Henry et al., 1910). By the 1980s over 1,500 UV facilities were installed to treat groundwater or bank-filtered water. Today, UV is a viable alternative to chlorine-based disinfection with hundreds of full-scale treatment plants operating in Ontario alone.

However, UV is not without its difficulties. For example, UV relies on the transmission of light to microorganisms. This light may be shielded or reflected by suspended solids, which decrease the efficiency of treatment. Fluctuations in wastewater flows and changes in the concentration of suspended solids can make achieving effluent standards a challenge (Caron et al., 2007; Li et al., 2009). Recently, researchers have found increased efficiencies when two disinfectants are used in combination or sequence (Cho

et al., 2011, 2006a; Jung et al., 2008; Koivunen and Heinonen-Tanski, 2005; Murphy et al., 2008; Pennell et al., 2008; Rennecker et al., 2000; Vankerckhoven et al., 2011). The research presented in this thesis aimed to study the sequential or combined use of UV with a chlorine-free disinfectant. Chlorine-free chemicals were selected to be paired with UV to reduce the potential of forming halogenated DBPs.

The following offers a review of the literature, first, of the individual chlorine-free wastewater disinfectants used in the study including UV, ferrate(VI), PAA and HP. Then, research that has investigated the combined or sequential use of two disinfectants is presented as well as the current status of the understanding of the VBNC-state of bacteria.

2.1 Ultraviolet light

The germicidal effects of UV have been known since the early 20th century (US EPA, 2003) and have been applied to air (Memarzadeh et al., 2010a), food (Koutchma, 2008), and of course, water (Guo et al., 2009b; Hassen et al., 2000; Murphy et al., 2008; US EPA, 2003). As wastewater disinfection technology, UV has many desirable qualities. For example, it limits the release of chemical residuals to the environment and eliminates the potential formation of DBP that are associated with chlorine, ozone or other chemical oxidants (US EPA, 2003). It has also been shown to be more effective at reducing chlorine-resistant microorganisms such as *Cryptosporidium parvum* oocysts and *Giardia lamblia* (Bukhari et al., 1999; Liberti et al., 2003; Linden et al., 2001). UV fluences required to inactivate *Cryptosporidium parvum* oocysts and *Giardia lamblia*

cysts have been shown to be 80% lower than those required for total coliforms (Tchobanoglous et al., 2003).

When microorganisms absorb UV in the UV-C (215-315nm) portion of the electromagnetic spectrum (see Table 2.1) photochemical changes occur to the nucleic acid of cells that prevent replication and therefore render the cells unable to cause infection. The most frequent change to occur is the dimerization of pyrimidine bases, where pyrimidine bases bond to each other instead of to their complementary purine bases (US EPA et al., 2006). The UV germicidal wavelength is considered to be 253.7 nm, the output wavelength of low pressure (LP) UV lamps. However, each microorganism will absorb UV at a different optimal wavelength. For example, *E. coli* has a peak absorbance at 265 nm (see Figure 2.1), while that of *B. subtilis* is 270 nm, and that of *Cryptosporidium parvum* oocysts is 271 nm (Bukhari et al., 1999; Dell'Erba et al., 2007; Liberti et al., 2003; Linden et al., 2001; Pang et al., 2007).

Table 2.1: Electromagnetic Spectrum

Type of Light	Colour	Wavelength	Application
UV-Vacuum		200-100 nm	
UV-C		280-200 nm	Low-P UV, germicidal UV at 253.7 nm, eye and skin irritant but not carcinogenic
UV-B		315-250 nm	Portion of solar UV that is dangerous/carcinogenic
UV-A		400-315 nm	Long wave UV, Black Light UV
Visible	Violet	380-450 nm	
	Blue	450-495 nm	
	Green	495-570nm	
	Yellow	570-590 nm	
	Orange	590-620 nm	
	Red	620-750 nm	

UV can be delivered by LP or medium-pressure (MP) mercury-vapour lamps that differ by spectrums patterns emitted. As mentioned above, LP lamps produce near the monochromatic light at 254.7 nm. MP lamps provide polychromatic light with various peaks ranging from 240 to 350 nm, see Figure 2.1 (Kowalski, 2009). The quantity of irradiation received by a sample is measured by the UV fluence (mJ/cm^2), which is the product of radiation intensity (mW/cm^2) and contact time (s) (Bolton and Linden, 2003). It is difficult to make a direct comparison between the effectiveness of LP and MP; however, Shin and Lee (2010) studied the effects of LP-UV and MP-UV on the inactivation of adenovirus. Using a dose of $40 \text{ mJ}/\text{cm}^2$ a 1-log reduction was achieved with LP lamps while 3-log reduction was observed with MP lamps (Shin and Lee, 2010). Adenovirus is particularly difficult to inactivate with UV disinfection, and the US EPA has

reported that an LP-UV fluence 186 mJ/cm^2 was required to achieve a 4-log reduction (US EPA et al., 2006) however, a dose less than 100 mJ/cm^2 was required when using MP-UV (Eischeid et al., 2009). Although MP-UV may have a broader spectrum and increased effectiveness, LP-UV remains more widely used.

Figure 2.1: Germicidal effectiveness of low and medium pressure UV lamps of E. coli. Reprinted with permission of Springer from UVGI Disinfection Theory, "Germicidal efficiency of UV wavelengths, comparing High (or medium) and Low pressure UV lamps with germicidal effectiveness for E. coli. Based on data from Luckiesh (1946) and IESNA (2000)", 2009, p. 18, Kowalski, W © Springer-Verlag Berlin Heidelberg 2009

Microorganisms damaged by UV have two primary repair mechanisms: photoreactivation and dark repair. Photoreactivation is the repair of pyrimidine dimers when organisms are exposed to light between 310 nm and 500 nm (WEF, 1996).

Photoreactivation enzymes form a complex with pyrimidine dimers, which are then catalyzed by absorbed light to create monomers and reverse the damage caused by UV (WEF, 1996). Dark repair occurs at a slower rate. It relies on enzymes to identify and excise dimers (WEF, 1996). Quek and Hu (2008) observed that when compared to LP-UV, lower reactivation rates occurred with MP-UV five hours after exposure. Guo et al., (2009a) investigated photoreactivation four hours after UV exposure of *E.coli* in wastewater and observed that an LP-UV fluence of 5 mJ/cm² achieved 50% photoreactivation while the same MP-UV fluence achieved 20% photoreactivation. When the fluence was increased to 40 mJ/cm², a 1% photoreactivation was observed after both LP-UV and MP-UV (Guo et al., 2009a). These studies suggest that photoreactivation at fluences above 40 mJ/ cm² are independent of the type of UV employed. LP-UV lamps are used throughout this work.

UV relies on the transmission of light reaching microorganisms and maintaining exposure for a prescribed amount of time and can be hindered by changes to UV transmittance or increases in suspended solids (US EPA et al., 2006). The use of a secondary disinfectant may be a way of partially mitigating some of these disadvantages. In subsequent sections, an overview of the chlorine-free chemicals selected is presented. Selection of these chemicals considered the potential change the transmittance or the turbidity of wastewater as well as possible secondary benefits that may occur due to the presence of UV. For example, the potential advantages of forming hydroxyl radicals which may occur when PAA and HP are exposed to UV.

2.2 Ferrate(VI)

Ferrate(VI) is a strong oxidant with an oxidation-reduction potential (ORP) of 2.2 volts at pH below 8 (0.72 volts at pH above 8), see Equation 2.1, which is similar to the ORP of ozone at 2.07 volts (Jiang et al., 2001). The effectiveness of ferrate(VI) is due to the +6 valence state, which is oxidized to ferric oxides and hydroxides containing iron that has a +3 valence state and can act as a coagulant, see Equations 2.2-2.3 (Farooq and Bari, 1986; Jiang et al., 2009, 2006; Murmann and Robinson, 1974). Researchers have investigated the use of ferrate(VI) for different stages of wastewater treatment as well as the use of ferrate(VI) as a combined disinfectant and coagulant (Bandala et al., 2009; Gombos et al., 2012; Jiang et al., 2009, 2006; White and Franklin, 1998). It has also been suggested for applications in agriculture (Bandala et al., 2009), wetland and groundwater restoration (Reimers et al., 2007) and ballast water (Jessen et al., 2006).

As a wastewater disinfectant, ferrate(VI) is an attractive candidate because it does not create halogenated DBPs associated with chlorine-based disinfectants (Schuck et al., 2006). In fact, ferrate(VI) has been shown to oxidize emerging contaminants of concern

such as antibiotics (Sharma et al., 2006), endocrine disrupting compounds (Li et al., 2008; Srisawat et al., 2010; Yang et al., 2012) and other pharmaceuticals (Jiang et al., 2013) as well as cyanide-containing compounds (Sharma et al., 2008), and other organic compounds (Avisar et al., 2010; Lee and von Gunten, 2010). The colour of ferrate(VI) and oxidized ferrate(VI) in solution may increase the UV-transmittance of wastewater and consequently change the UV fluence calculations.

As previously mentioned, ferrate(VI) is reduced in aqueous solutions to ferric compounds containing Fe(III), which act as a coagulant (Bandala et al., 2009; Sharma, 2002). Consequently, ferrate(VI) can simultaneously serve as a disinfectant and a coagulant. Jiang et al. (2009) found that concentrations of 0.005 to 0.04 mg/L as Fe(VI) were able to achieve similar suspended solid, phosphate, and COD removals as a ferric sulfate dose of 37 mg/L (Jiang et al., 2009). A study by Gombos et al. (2012) compared the effects of chlorine and ferrate(VI) on the indigenous bacterial community of secondary effluent wastewater. The authors found that 5 mg/L as Fe(VI) and a contact time of 30 minutes was sufficient to achieve a 4-log reduction in bacteria. Ferrate(VI) dosages and contact times were smaller and shorter than those required for chlorine gas to reach similar levels of disinfection (Gombos et al., 2012). Bandala et al. (2009) investigated the use of ferrate(VI) as a disinfectant and achieved a 4-log reduction in fecal coliforms with a dose of 5 mg/L as Fe(VI). Ferrate(VI) can also achieve 28-48% reduction in organic matter, depending on applied concentration (Bandala et al., 2009). Jiang et al. (2006) investigated the use of ferrate(VI) as a substitute for the combined

use of sodium hypochlorite (NaOCl) and ferric sulfate or alum for drinking water and wastewater treatment. For drinking water, the study found that 6 mg/L as Fe(VI) achieved a 6-log reduction in *E. coli* and an additional 10-20% reduction of dissolved organic matter (Jiang et al., 2006). For wastewater, ferrate(VI) achieved an additional 3-log reduction in bacteria and 30% reduction in COD. It also produced lower sludge volumes (Jiang et al., 2006).

2.3 Hydrogen peroxide

HP is the simplest compound to contain the peroxide molecule; a molecule composed of two oxygen atoms joined by a single bond. In theory, HP (H_2O_2) is an ideal disinfectant. It is readily available as a liquid and is stable in a wide variety of concentrations. It also has a high ORP of 1.78 V or 1.3 times that of chlorine. In practice; however, HP does not perform as expected. For example, Wagner et al. (2002) evaluated the use of HP for the disinfection of effluent wastewater and found that doses between 100 and 250 mg/L with a contact time of 2 hours would be required to reduce fecal coliform concentrations from 10^6 CFU/100mL to 10^4 CFU/100mL; a difference of up to a factor of 20 when compared to dosages of chlorine needed to achieve same level of disinfection (Tchobanoglous et al., 2003).

When HP is exposed to UV between 200 and 280 nm, an advanced oxidation process (AOP) is created producing two hydroxyl radicals ($OH^\bullet$). With an ORP of 2.80 V, the hydroxyl radical is 1.6 times stronger than HP and 2.05 times that of chlorine (Tchobanoglous et al., 2003). The hydroxyl radical is non-selective and can mineralize

chemical compounds as well as cellular components. The combination of HP and UV has been used successfully to degrade precursors of DBPs (Chen et al., 2010). Cho et al. (2011) observed that the addition of HP to LP-UV followed by free chlorine enhanced the inactivation of *B.subtilis* spores an additional 1.3- to 1.5-logs. Vankerckhoven et al. (2011) observed a further reduction in bacteria of 3.1-logs when 5 ppm HP was used in addition to UV.

2.4 Peracetic acid

PAA ($\text{CH}_3\text{CO}_3\text{H}$) is the peroxide of acetic acid and exists in equilibrium solutions with acetic acid, and HP, see Equation 2.4. Commercial solutions are available at concentrations ranging between 1 and 15% PAA, while 12% is most commonly seen (Kitis, 2004).

Where CH_3CO_2 is acetic acid (vinegar), H_2O_2 is hydrogen peroxide and $\text{CH}_3\text{CO}_3\text{H}$ is peracetic acid (Tchobanoglous et al., 2003)

PAA has an oxidation potential of 1.76 volts, which is similar to that of HP at 1.78 volts. However, disinfection with PAA requires much lower dosages and is considered a more viable alternative to chlorine. A study comparing the use of HP and PAA as a disinfectant found that 100 mg/L of H_2O_2 with a 2-hour contact time or 1.6 mg/L of PAA with a

contact time of 30 minutes would be required to achieve a 2-log reduction in fecal coliforms (Wagner et al., 2002).

Similar to HP, hydroxyl radicals are created when PAA is exposed to UV, and the sequential use of PAA and UV has been shown to have synergistic effects; meaning an additional disinfection capacity was created and was explained by the formation of hydroxyl radicals. Koivunen and Heinonen-Tanski (2005) observed an increased disinfection efficiency when PAA was followed by UV for the disinfection of wastewater. Caretti and Lubello (2003) examined the use of peracetic added before and after UV and found that while both regimes offered increased efficiency, gains were greater when PAA was added upstream of UV. This effect was explained by the formation of hydroxyl radicals. An additional reduction in bacteria of 0.8 logs was observed when 2.3 ppm PAA was added to UV disinfection of a pilot-scale study (Vankerckhoven et al., 2011).

As a disinfectant, PAA requires short contact times and is effective over a pH range from 1 to 10, and a temperature range from 0°C to 100°C. It has been shown to be effective as a bactericide and virucide (Lazarova et al., 1998; Rajala-Mustonen et al., 1997; Wagner et al., 2002). Reaction products include acetic acid (CH_3COOH), oxygen (O_2), methane (CH_4), carbon dioxide (CO_2), and water (H_2O), all of which are produced at nontoxic concentrations (Tchobanoglous et al., 2003). However, the lag time required to generate the non-toxic by-products has been shown to depend on the organic and inorganic load of a sample (Antonelli et al., 2009). A study by Monarca et al. (2002) found that the main reaction products of PAA were carboxylic acids, a functional group

that includes acetic acid. In a pilot study, Dell'Erba et al. (2007) found no evidence of aldehydes or halogenated phenols, two common DBP groups associated with the use of chlorine, in secondary wastewater treated with PAA. A study by Lazarova et al. (1998) found that acetic acid significantly increased the pollution load of treated wastewater, as measured by an increase in the biodegradable dissolved organic carbon (BDOC). The previously mentioned study by Lazarova et al. (1998) also demonstrated a decrease of culturable cells after disinfection with PAA, which was accompanied by a smaller decrease in cell respiration and enzyme activity of β -D-galactosidase. After diluted with fresh water and incubation for 2-days, bacterial quantities had returned to levels before disinfection indicating regrowth. Rossi et al. (2007) observed no significant regrowth in the 5 hours following disinfection with PAA, and Collivignarelli et al. (2000) found an increase in COD between 5 and 6 mg/L with wastewater due to the addition of PAA and the acetic acid by-product.

The quantity and size distribution of total suspended solids in a sample has been shown to affect both chemical disinfection with chlorine chemicals and physical disinfection with UV. Particle-associated bacteria (PAB) are protected from disinfectants, and additional chemical dosages may be required to diffuse into particles or shielding may prevent UV from reaching the bacteria. Falsanisi et al. (2008) used wastewater to determine the effect of total suspended solids, as well as particle size, on the effectiveness of disinfection with PAA. The authors found that the TSS of a sample affected bacterial inactivation by up to 2-logs. Also, larger particles of 120 μ m offered up

to twice as much protection than smaller particles ranging between 10 μm -120 μm . The sequential use of UV and PAA may be adversely affected by turbidity. Rajala-Mustonen et al. (1997) showed that an increase in turbidity from 2 to 10 NTU decreased the performance of disinfection with PAA and UV with a loss of 2-log reduction in bacteria.

2.1 Combined and sequential disinfection treatment

Sequential, or combined, disinfection is the application of two or more disinfection methods. The purpose of applying two disinfectants is to increase disinfection efficiencies, to take advantage of synergistic effects or to take advantage of two disinfection mechanisms. For example, chlorination is often performed following UV disinfection to provide additional assurance as to the reduction of indicator organism while minimizing the use of chlorinated-compounds and the formation of halogenated DBPs (Wang et al., 2012).

Within the literature, the terms synergisms, efficiencies, and disinfection gains are often used interchangeably and have been defined in different ways. Mathematically, Kouame and Haas (1991) defined synergism when “a combination of agents is more effective than [what] is expected from the single component effectiveness of its constituents”, see Equation 2.5. This definition has also been used by Dykstra et al. (2007). Following equation 2.5, Kouame and Haas (1991) observed synergistic effects when free chlorine and monochloramine were used in combination for the inactivation of *E. coli*.

$$\sum_{i=1}^n \frac{x_i}{y_i} \quad 2.5$$

Where x_i is the concentration of the individual agent in the combination, y_i is the concentration of the agents that individually would produce the same magnitude of effect as that of the combination, i is the individual agent and n is the total number of agents (Kouame and Haas, 1991). If the sum is < 1 then synergistic interactions are observed, > 1 , antagonistic interactions are observed and $= 1$ then zero interactions are observed.

Equation 2.5 defines a synergistic effect as being greater than additive. However, additivity assumes a linear relationship between the concentration of a disinfectant and the removal of enteric organism, which is not necessarily accurate especially at higher log removals. Typically in disinfection, the relationship between concentration of a disinfectant and the removal of enteric organisms is non-linear. For example, marginal gains in the reduction of coliform bacteria are seen with UV fluences above 40 mJ/cm² are applied. This is known as the tailing effect and is typically explained by the presence of particle associated bacteria that are shielded from UV. As such, the effect of adding a second disinfectant in combination or in sequence to UV is not expected to be additive. It may be more appropriate to speak in terms of “gains” or “improvements” in treatment compared to an individual treatment.

A number of combined or sequential disinfectant pairs have been found in the literature. For example, Rennecker et al. (2000) improved the inactivation of *Cryptosporidium parvum* with the sequential use of ozone with free chlorine or

monochloramine. In a different study, Cho et al. (2006) investigated the use of ozone, chlorine dioxide or UV followed by free chlorine for disinfection of *Bacillus subtilis* (*B.subtilis*) spores. The most effective regime was found to be chlorine dioxide followed by free chlorine and no added benefit was found when UV was used as primary disinfectant (Cho et al., 2006).

Combinations that include UV have also been shown to have beneficial effects. Jung et al. (2008) examined combinations of UV and ozone for the inactivation of *B.subtilis*. The authors found that the most effective regime was the addition of ozone before UV due to the photolytic formation of hydroxyl radicals (Jung et al., 2008). The reverse treatment of UV followed by ozone did not show any added advantage as hydroxyl radical were not formed (Jung et al., 2008). Similarly, PAA may also create oxidative species such as single oxygen, ozone, and hydroxyl radicals when exposed to UV (Caretti and Lubello, 2003; Koivunen and Heinonen-Tanski, 2005; Vankerckhoven et al., 2011). Koivunen and Heinonen-Tanski (2005) observed an increase in disinfection efficiency when UV was followed PAA for the disinfection of wastewater. Caretti and Lubello (2003) examined the use of PAA added before and after UV and found that both regimes offered increased efficiency; however, gains were greater when PAA was added upstream of the UV reactor suggesting greater efficiency was due to the formation of oxidative species. In a separate study, an additional 0.8 Log-reduction of bacteria were observed when 2.3 mg/L PAA was added to a pilot-scale UV disinfection treatment system in Belgium (Vankerckhoven et al., 2011).

The combined use of UV and HP has also been investigated. Cho et al. (2011) showed that HP enhanced disinfection with UV due to the formation of hydroxyl radicals. The treatment was also shown to degrade selected emerging contaminants such as including the endocrine disrupting compound bisphenol-A and herbicide 2,4-D. Koivunen and Heinonen-Tanski (2005) showed that the effectiveness of UV/HP treatment was less than that of UV/PAA treatment. Other UV-free alternatives have also been investigated showing favorable results. Cho et al. (2006) showed that the sequential use of chlorine dioxide and free chlorine could further reduce concentrations *B. subtilis*. Beneficial effects have also been observed when chlorine was combined with ozone (Rennecker et al., 2000).

A study by Cho et al. (2011) investigated the disinfection mechanism of different disinfection methods. The study found that ozone, a strong oxidant, had a greater effect with the cell on the cell wall and caused protein release, lipid peroxidation, and changes in membrane permeability (Cho et al., 2011). Free chlorine, a less reactive oxidant, was shown to affect internal cell components, such as enzyme, suggesting that it was able to diffuse into the cell wall (Cho et al., 2011). The study also looked at UV, which is a physical disinfection method that induces changes to the nucleic material of a cell. As such, UV had no effect on surface structures and internal cell components remained unchanged (Cho et al., 2011).

The combination of UV with ozone, PAA, or HP has been shown to increase disinfection efficiencies due to the formation of hydroxyl radicals and other combinations may also

offer synergisms. For example, Pennell et al. (2008) found that the use of UV followed by iodine increased inactivation levels of *B. subtilis*. It was hypothesized that UV applied first increased the susceptibility of *B. subtilis* to the iodine. Cho et al. (2006) examined the inactivation of *B. subtilis* after ozone, chlorine dioxide, or UV followed by free chlorine. Interestingly, the study found that UV was the least effective when used first. The most effective combination was found to be chlorine dioxide followed by free chlorine. The authors proposed that the success of the treatment relied on the ability of the first disinfectant to break down the protein coat of spores allowing the transport of the second disinfectant to the inner cell. To this end, ozone, and chlorine dioxide was the most effective at degrading the protein coat. The effectiveness of the sequential use of chlorine dioxide and free chlorine was further reasoned to be due to the ability of chlorine dioxide to consume reaction sites on lipid components of the inner cell that may otherwise be attacked by free chlorine allowing for a greater synergistic effect for the use of this combination of disinfectants (Cho et al., 2006a). The effectiveness of certain treatments may be complex and vary depending on the disinfection pair as well as the test organism.

2.2 Viable but nonculturable bacteria

Culture methods have long been the gold standard to assess the effectiveness of wastewater disinfection. Typically, non-pathogenic indicator organisms are cultured on selective media and colonies are counted to determine the level of contamination of a sample after treatment (World Health Organization and Organisation for Economic Co-

operation and Development, 2003). However, the absence of indicator organisms does not account for the presence of more resistant viruses or protozoa nor does it account for bacteria that are viable but nonculturable (VBNC), which may occur due to low-nutrient conditions or other stresses such as solar radiation or chemical disinfection.

Bacteria may lose culturability but retain metabolic activity a pseudo-dormant physiological state termed VBNC (Calgua et al., 2014; Oliver, 2005; Oliver et al., 2005; Xu et al., 1982). Xu et al., (1982) first described the VBNC state when they observed that *E. coli* and *Vibrio cholera* loss culturability after incubation in saltwater but maintained viable characteristics such as positive substrate-response using the direct viable count (DVC) method. Since then, over 60 bacterial strains have been shown to exist in a VBNC-state (Oliver, 2010, 2005), the induction of which is considered to be a survival response to stressors such as nutrient starvation (K. L. Cook and Bolster, 2007), exposure to low temperatures (Maalej et al., 2004; Wong and Wang, 2004), low oxygen conditions (Kana et al., 2008), and exposure to white as well as sunlight (Besnard et al., 2002; Gourmelon et al., 1994).

In the context of wastewater disinfection, the concern is that undetected VBNC bacteria are being released to the environment and could cause infection further downstream. Oxidative stress due to chemical disinfection has been shown to induce the VBNC-state (Noor et al., 2009; Oliver et al., 2005; Servais et al., 2009). For example, Oliver et al. (2005) studied different states of *E. coli* and *Salmonella typhimurium* after exposure to chlorine and found that 10^4 cells/mL of the initial 10^6 cells/mL survived in a VBNC state

as determined by DVC. Also, mixing treated wastewater with nutrient deficient receiving water may also play a role in maintaining cells in a VBNC-state. When introduced into a low-nutrient environment, such as drinking water, Byrd et al. (1991) observed a loss of culturability of three gram-positive bacteria (*M. flavus*, *S. feacalis*, *B. subtilis*) and five gram-negative bacteria (*A. Tumeefaciens*, *E. aerogenes* and *K. pneumoniae*, *Pseudomonas* sp. strain 719 and *P. fluorescens* L-2). Bacteria were also shown to remain in this state for periods extending beyond 95 days (the duration of the experiment) (Byrd et al., 1992). A similar situation was observed following wastewater treatment. A study by Servais et al. (2009) compared quantities of culturable and viable *E. coli* cells in freshwater samples taken near and at a distance from wastewater treatment plants. The study found that samples near wastewater effluent mixing points had viable and culturable bacteria while samples collected further downstream or taken from shallow bodies of water had a higher number of VBNC bacteria induced by a nutrient-limited environment and stress from solar radiation (Servais et al., 2009).

The most commonly used methods to detect VBNC bacteria are DVC, live/dead viability stain, and reverse transcriptase-polymerase chain reaction (RT-PCR), which can all have limitations in natural and wastewater samples (Oliver, 2010). A recent molecular method combining the use of DNA-intercalating dye propidium monoazide (PMA) and quantitative polymerase chain reaction (qPCR) has been developed and used for dilute and complex matrices such as drinking water (Zhang et al., 2015), wastewater (Bae and

Wuertz, 2009; Varma et al., 2009) and sludge samples (van Frankenhuyzen et al., 2013, 2011).

Following chlorine disinfection of secondary wastewater, Oliver et al. (2005) observed a small percentage (0.4%) of bacteria cells were able to avoid death and survived in a VBNC state. Bacteria examined included *E.coli* and *Salmonella Typhimurium* (Oliver et al., 2005). When introduced into a low-nutrient environment, such as treated drinking water, Byrd et al. (1992) observed a loss of culturability of three gram-positive (*M.Flavus*, *S.feacalis*, *B.Subtilis*) and five gram-negative (*A. tumefaciens*, *E. aerogenes* and *K. pneumoniae*, *Pseudomonas sp.* strain 719 and *P. Fluorescens* L-2) bacterial strains. Bacteria were also shown to be able to remain in this state for periods extending beyond 95 days, the duration of the experiment (Byrd et al., 1992). A similar situation may be observed following wastewater treatment, where nutrients are significantly reduced. A study by Servais et al, (2009) compared the quantities of culturable and viable *E.coli* cells in freshwater samples taken near and at a distance from wastewater treatment plants. The study found that water samples collected near wastewater effluent mixing points had viable and culturable bacteria (Servais et al., 2009). Samples collected further from effluent mixing points or taken from shallow bodies of water had a higher number of VBNC induced by limited nutrients and solar radiation (Servais et al., 2009). These results imply that bacteria released into natural water sources may become VBNC due to low-nutrient conditions, but if conditions change, cells may become culturable again.

2.3 Conclusions

In the interest of studying the individual and combined use of UV with chlorine-free chemicals the literature review outlined the current state of knowledge of each disinfectant considered both alone and if it has been previously considered in combined disinfection studies.

The combination of UV and chlorine-free chemicals is a disinfection strategy that reduces if not eliminates the formation of halogenated DBP and may provide synergisms that would further increase disinfection capacity. Of the individual disinfectants selected, ferrate(VI) residuals also may further reduce suspended solids of a sample, and the use of HP and PAA may form oxidative species that could increase disinfection capacity and oxidize emerging contaminants of concern. Of the disinfectants selected, none have shown to produce halogenated DBPs and disinfection residuals have been shown to be non-toxic at concentrations produced. However, not all combinations are expected to be equally effective. The effectiveness of treatment relies on the disinfection mechanisms of the pairing as well as the test organisms selected.

Many of the studies listed above have evaluated the performance of treatments using culture methods. To further understanding the effectiveness of the disinfection pair, a variety of test organisms with different characteristics has been used throughout the study. Also, the review has shown that the loss of culturability does not necessarily lead to a loss of viability. Incorporating the use of molecular methods to further investigate

the VBNC-state of bacteria following disinfection is a research gap that is in need of being filled in the field of environmental engineering.

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Chapter 3

3 Materials and Methods

This chapter describes the materials and methods developed and employed for the completion of the thesis and are intended to act as a guide for future students performing similar lab work. Subsequent research chapters (Chapters 4-7) also include materials and methods sections required for submission to journals.

3.1 Disinfection with ultraviolet light

The laboratory is equipped with a bench-top UV collimated beam apparatus, and experiments with UV irradiation were performed as batch experiments (see Figure 3.1). Samples were placed in a Petri-dish and stirred magnetically with a micro stir bar during irradiation. The bottom aperture was covered while the lamps are turned on to allow for the light source to stabilize. The aperture was then opened while a timer that was pre-set to the time required for irradiation was started simultaneously.

Figure 3.1: UV collimated beam apparatus and set up. Reprinted with permission from ASCE from Bolton, J. R., Linden, K. G., 2003. Standardization of methods for fluence (UV dose) determination in bench-scale UV experiments. *J. Environ. Eng.* 129, 209-215. Doi: 10.1061/(ASCE)0733-9372(2003)129:3(209) © 2003 ASCE

3.1.1 Ultraviolet light fluence calculation

The fluence, or UV dose, is measured in J/m^2 or mJ/cm^2 and is the total amount of radiant power delivered to the surface area of an infinitesimally small sphere, for a specific amount of time. The fluence is defined as:

$$\text{Fluence} = E'_{\text{avg}} \times t \quad 3.1$$

Where E'_{avg} [W/m^2] is the fluence rate and t [s] the exposure time (Bolton and Linden, 2003)

To apply Equation 3.1, the desired fluence is selected, and the average fluence rate is calculated using a series of equations (shown below). The time of exposure is then determined to provide the desired fluence to the sample. As a frame of reference, a typical water treatment plant is designed to deliver a fluence of 40 mJ/cm² while a wastewater treatment plant aims to deliver 60 mJ/cm².

The average fluence rate is defined as followed:

$$E'_{avg} = E_0 \times F_{Petri} \times F_{reflection} \times F_{water} \times F_{divergence} \quad 3.2$$

Where E_0 is the radiant power [W/m²], F_{Petri} [unitless] is the Petri factor, $F_{reflection}$ [unitless] the reflection factor, F_{water} [unitless] is the water factor and $F_{divergence}$ [unitless] is the divergence factor (Bolton and Linden, 2003).

The series of factors, shown in Equation 3.2, aim to correct the measured radiant power to what is more accurately delivered to the sample. The radiant power was measured with the International Light NIS Traceable Radiometer/Photometer (Model IL 1399A) at the same height as the surface of the liquid to be irradiated. The Petri factor is the average irradiance delivered over the surface area of the sample and corrects for the variations of irradiance that may occur over the liquid's surface (Bolton and Linden, 2003). Due to the small surface area of the sample, there were no observed differences in the irradiation field, and the Petri factor was set to 1 for the duration of testing. The reflection factor accounts for the portion of light that is reflected as it hits the surface of

the liquid. For UV light between 200 and 300 nm, the amount of light reflected is equal to $R = 0.025$. The corresponding reflection factor is equal to $1-R$ and is thus equal to 0.975 (Bolton and Linden, 2003). The water factor accounts for UV light that is absorbed by water and thus not delivered to the sample. The water factor is defined as:

$$F_{water} = \frac{1-10^{-al}}{al \ln 10} \quad 3.3$$

Where a is the 1-cm absorbance at 254 nm of a sample [cm^{-1}] and l the vertical path length [cm] or depth of water in the Petri dish.

Throughout testing the vertical path length was maintained at or below 2 cm to maintain shallow dish configuration. The absorbance was measured before every experiment with the UV-Visible spectrophotometer (Varian Model Cary 100BIO) using a 1-cm quartz cuvette. The spectrophotometer was allowed to warm up for approximately 15 minutes before use and was first zeroed with deionized water. The divergence factor is defined as:

$$F_{divergence} = \frac{L}{(L+l)} \quad 3.4$$

Where L is the distance between the UV lamps and the surface of the sample [cm].

Throughout testing, a similar style of Petri dish and water depth was maintained to keep L consistent. If any elements in the experimental set-up were changed, then L was re-measured. The variable l is the vertical path length and has been previously described above (Bolton and Linden, 2003). The fluence delivered to a sample can thus be calculated by considering all the factors and placing them into Equation 3.2.

3.2 Chemical disinfection

Chemical disinfection experiments were performed as batch experiments using a jar test apparatus with continuous mixing at 70 rpm. All experiments were conducted in sterile glass beakers which were covered in foil to protect against light exposure. Chemical dosages were calculated and measured using the appropriate method described in subsequent sections. Quenching of chemical treatments was performed after the contact time had been reached and assured by measuring residual concentrations of 0 mg/L. Quenching protocols are described below.

3.2.1 Sodium hypochlorite

Chlorination was performed using 5.25% sodium hypochlorite (NaOCl) without any further pH adjustments. Throughout testing total and free chlorine was measured using HACH methods 8167 with N,N-Diethyl-p-Phenylenediamine (DPD) Total Chlorine Reagent and DPD Free Chlorine Reagent (Hach Permachem Reagents, Mississauga ON) in conjunction with the Hach DR2800 Spectrophotometer. A 1 g/L stock solution was prepared weekly and the concentration of which was assured by testing free and total chlorine concentrations using the above mentioned HACH method. The stock solution

was stored at 4°C and was protected from light in a glass jar covered with aluminum foil. Once the contact time had elapsed, dechlorination or quenching was performed to eliminate chlorine residuals from further affecting bacteria concentrations. Quenching was conducted with a 0.02% (w/v) solution of Sodium Thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3$) (Fisher Scientific, New Jersey NY). A weight ratio of 0.6-0.9 mg of sodium thiosulfate per mg/L of chlorine residual was used as an initial estimate of the quantity of sodium thiosulfate required (Tchobanoglous et al., 2003). Quenching was assured by measuring a chlorine residual of 0 mg/L after 2 minutes of mixing.

3.2.2 Ferrate(VI)

Potassium ferrate, K_2FeO_4 , is a stable and commercially available ferrate salt. In solution creates an equilibrium relationship between iron ions of high oxidation states such as ferrate(VI) or $[\text{FeO}_4]^{2-}$ and ferrate(V) or $[\text{FeO}_4]^{3-}$. Ferrate(VI) is the most stable above pH of 9 (Lee et al., 2005; Sharma, 2013). Ferrate(VI) stock solutions were created by dissolving of 1 g/L of potassium ferrate(VI) salts, K_2FeO_4 , (Sigma-Aldrich, MO, USA, >90%) in ultrapure water (Millipore Direct-q UV 3). While some researchers have dissolved ferrate in buffer solutions (Casbeer et al., 2013) others have reported that when diluted in distilled water the pH was maintained above 9 and assured stability (Lee et al., 2005). Dissolving ferrate(VI) in a buffer containing 10 mM KOH can increase the stability of the solution to weeks (Tiwari and Lee, 2011). The pH of the stock solution was measured to be 11.16 and was considered stable. Solutions were stored in pre-sterilized dark glass bottles at 4°C until use.

Solutions of potassium ferrate(VI) are violet in colour with a characteristic peak at 510 nm and decompose to produce ferrate(V), which has a much different absorption profile, see Figure 3.2 (Bielski and Thomas, 1987; Sharma, 2013; Tiwari and Lee, 2011). The speed at which the decomposition occurs can depend on temperature, exposure to light and pH. To determine the speed of the decomposition of ferrate(VI) stock solutions degrade within the conditions of the lab, the visible absorption spectra of a 10 fold dilution of the ferrate(VI) stock solution was measured with time, see in Figure 3.3. The initial curve demonstrates the characteristic peak at 510 nm and a valley at 390 nm. A smoothing of the curve with time is observed and by 24 hours the curve resembles that of ferrate(V) as seen in Figure 3.2. The conversion rate of ferrate(VI) to ferrate(V) was determined by plotting the peak absorbance ($\lambda = 510$ nm) of the solution with time, see Figure 3.4. A 10% loss of ferrate(VI) was observed at 3 hours, and a 30% loss was observed at 8 hours. To maintain starting conditions, a new stock solution was made every 3 hours, as required.

Figure 3.2: Visible Absorption spectra of Iron Ferrates in Fe(VI) (FeO_4^{2-}) and Fe(V) (FeO_4^{3-}) showing the characteristic peak of Fe(VI) at 510 nm. Reprinted with permission from Bielski, B.H.J., Thomas, M.J., 1987. Studies of hypervalent iron in aqueous solutions. 1. Radiation-induced reduction of iron(VI) to iron(V) by CO_2 . J. Am. Chem. Soc. 109, 7761-7764. Doi: 10.1021/ja00259a026 © 1987 American Chemical Society.

Figure 3.3: Visible absorption spectra of a 10 fold dilution of 1g/L potassium ferrate(VI) stock solution with time

Figure 3.4: Conversion of ferrate(VI) to ferrate(V) in solution with time

Figure 3.5 demonstrates the speciation of the ferrate(VI) ion with pH. The pKa of the protonated form of the ferrate(VI) (HFeO_4^-) and the un-protonated form (FeO_4^{2-}) occurs between 7.2 to 7.3 (Sharma, 2008; Tiwari et al., 2007). The protonated form of ferrate(VI) is a more powerful oxidant however changing the pH of large volumes of wastewater could be problematic. The pH of wastewater was measured to be 7.6 ± 0.1 favoring the unprotonated form of ferrate(VI). However, similar *E. coli* levels as those reported in the literature were achieved and removal levels were consistent. No pH adjustments were made however; the efficiency of oxidation could be improved by lowering the pH.

Figure 3.5: Speciation of ferrate(VI). Reprinted with permission of Taylor & Francis from Tiwari, D., Kim, H., Choi, B., Lee, S., Kwon, H., Choi, K., Yang, J., 2007. Ferrate(VI): A green chemical for the oxidation of cyanide in aqueous/waste solutions, "Speciation of ferrate(VI) in aqueous solutions [Concentrations of Fe(VI): 1 mmol/L]". J. Environ. Sci. Heal. Part A 42, 803-810. Doi 10.1080/10934520701304674 © 2007 Taylor & Francis

Methods that have been used to measure the concentration of ferrate(VI) in solution and are summarized in Table 3.1 (Luo et al., 2011; Tiwari and Lee, 2011). Two colorimetric methods, the direct colorimetric method and the ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonate)) colorimetric method, were selected for evaluation based on ease and simplicity of the method, availability of equipment in the lab, suitability for use with aqueous samples, sensitivity of the method, and reaction products that did not have special disposal requirements. Both colorimetric methods selected are based on the Beer-Lambert Law, see Equation 3.5, which states that the absorbance is directly proportional to the concentration of substance multiplied by a proportionality coefficient called the molar absorptivity.

$$A = \epsilon \times l \times c \quad 3.5$$

Where A is the absorbance [unitless], ϵ is the molar absorptivity [$\text{L mol}^{-1} \text{cm}^{-1}$], l is the path length [cm] and c the molar concentration of the compound in solution [mol L^{-1}].

As the name implies, the direct colorimetric method measures the absorbance of ferrate(VI) in solution directly at the peak wavelength of 510 nm. The ABTS methods utilizes a colour change that occurs when ABTS reacts with ferrate(VI) in a 1:1 molar ratio to produce a radical, ABTS*, that is green and can be measured at the peak absorbance wavelength of 415 nm (Lee et al., 2005). To evaluate the methods, dilutions of the ferrate(VI) stock solution were prepared using deionized water. Visible absorption spectra (340-700 nm) scans were completed using a 1-cm path and the spectrophotometer (Varian Model Cary 100BIO) where the absorbance of deionized water was subtracted from all absorbance measurements. Calibrations curves were then created by plotting the appropriate peak absorbance versus concentration.

Table 3.1: Comparison of methods to measure ferrate(VI) in solution

Method	Description	Advantages	Disadvantages
Volumetric Titration Methods			
Titration with Arsenic(III) or chromate(VI)	Titrate standard ferrate solutions with sodium diphenylamine sulfonate indicator	Easy to perform, no specialized equipment required, can be used to measure solutions of sodium and potassium ferrate(VI)	Special disposal considerations, limited detection limits of approximately 1M
Electrochemical Method			
Potentiometry	Led wire electrode as indicator electrode in alkaline solution used with chromium(III) hydroxide indicator	Detection range of 1 mM Better accuracy compared to volumetric titration methods	Used for electro-generated ferrate species
Spectrophotometric Methods			
Fourier Transform Infrared (FTIR)	Reaction with BaSO ₄ , measure absorbance at 780 nm	Can determine the concentration of other species (not only Fe(VI)) in solution	Requires specialized equipment, requires preparation of KBr pellet
Mössbauer spectroscopy	Measures the recoilless gamma ray emission and absorption of atoms in solution	Useful in the determination of the oxidation states of iron +2 - +6 and can determine the % of Fe(VI) in a solution	Requires specialized equipment
Fluorescence	Reaction with scopoletin (fluorescent agent), excitation $\lambda = 335$ nm, emission $\lambda = 460$ nm	Less than 1 μ M detection limit, increased detection limits in alkaline solutions	Fluorescence varies with pH
Direct Colorimetric Method	Direct measurement of peak absorbance at 510 nm	Easy to perform, $\epsilon_{510\text{nm}} = 1.15 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$	Molar absorptivity of Fe(VI) changes with pH, Fe(VI) is most stable in alkaline solutions and dilutions in wastewater may make it unstable, colloidal Fe(III) may hinder absorbance
ABTS Colorimetric Method	Fe(VI) reacts with ABTS to create ABTS radical (ABTS*), which is green in colour. Peak absorbance at 415 nm	Better for low concentrations of Fe(VI), $\epsilon_{415\text{nm}} = 3.40 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$, ϵ is one order of magnitude higher than direct colorimetric method, suitable for aqueous solutions with pH >4.3	Harder to perform compared to direct colorimetric method, other products formed by reacting with ABTS may hinder absorbance
I ⁻³ Colorimetric Method	Reaction with 0.10 M sodium iodide (NaI), peak absorbance at 351 nm	Benign reagent, $\epsilon_{351\text{nm}} = 2.97 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$	Absorbance varies with pH, new method

Results of the direct colorimetric method and resulting calibration curve are presented in Figure 3.6 and Figure 3.7, respectively. The direct colorimetric method was found to be linear up to concentrations between 10 and 750 mg/L ($R^2 = 0.9998$), see Figure 3.7, but was not sensitive enough to measure concentrations below 10 mg/L as K_2FeO_4 (results not shown). Results of the ABTS method and the resulting calibration curves are presented in Figure 3.8 and Figure 3.9, respectively. An ABTS stock solution was created by dissolving 1 g/L ABTS in deionized water and stored at 4°C and reacted with ferrate(VI) in 1:1-mole ratio (Lee et al., 2005). Results demonstrate that the ABTS method was linear up to concentrations of 100 mg/L as K_2FeO_4 ($R^2 = 0.999$), see Figure 3.99-a, and was able to measure lower concentrations of ferrate(VI) between 1 and 10 mg/L as K_2FeO_4 ($R^2 = 0.9972$), see Figure 3.9-b. The direct colorimetric method was used to assure the concentration of the stock solution however for solutions of lower concentrations of ferrate(VI) the ABTS method was used.

Figure 3.6: Visible absorption spectrum of dilutions of ferrate(VI) solutions of 10 to 750 mg/L as K_2FeO_4

Figure 3.7: Calibration curve of the direct colorimetric method plotting absorption at 510 nm of ferrate(VI) solutions of 10 to 750 mg/L as K_2FeO_4

Figure 3.8: Visible absorption spectrum of dilutions of ferrate(VI) reacted with ABTS for (a) for ferrate(VI) solutions of 1 to 100 mg/L as K_2FeO_4 , and (b) for ferrate(VI) solutions of 1 to 10 mg/L as K_2FeO_4

Figure 3.9: Calibration curve of the ABTS method and dilutions of ferrate(VI) solution (a) for ferrate(VI) solutions of 1 to 100 mg/L as K_2FeO_4 , and (b) for ferrate(VI) solutions of 1 to 10 mg/L as K_2FeO_4

The ABTS method was also evaluated for use with wastewater, 10, for ferrate(VI) concentrations ranging between 1 and 25 mg/L as K_2FeO_4 . When dissolved in wastewater, the ABTS method was linear for concentrations between 2.5 and 25 mg/L as K_2FeO_4 ($R^2 = 0.9757$).

Figure 3.10: Measuring the concentration of ferrate(VI) in wastewater using the ABTS method for 1-25 mg/L as K_2FeO_4 (a) visible absorption spectra (b) resulting calibration curve

Quenching of ferrate(VI) in wastewater was performed with a solution of 0.02% (w/v) sodium thiosulfate and assured by using the ABTS method with an additional 10% sodium thiosulfate added (Bandala et al., 2009; Lee and von Gunten, 2010; Zhu et al., 2006).

3.2.3 Hydrogen peroxide

A household solution of 3% HP was used without further dilution throughout testing. Concentrations of HP were measured by using a colorimetric reaction between ABTS, horseradish peroxidase (HRP), and peroxide compounds such as HP and PAA (Wagner et al., 2002). To complete the ABTS-HRP protocol, a 10 mM ABTS solution with phosphate buffer (pH 6) and a 0.5 mg/mL HRP was prepared with deionized water. For a 7mL reaction, 0.7 mL of the ABT solution was added to 0.7 mL of the HRP solutions with the remaining volume composed of the desired quantity of HP diluted in phosphate buffer (pH 6) or wastewater. Peak absorbance at 405 nm was measured using the spectrophotometer (Varian Model Cary 100BIO) and results reported in Figure 3.11. Results demonstrated a linear relationship when solutions were diluted with either wastewater or the phosphate buffer. The limitation of the method is that HP concentrations must be below 1 mg/L or dilutions of HP solution are required before measurement. Quenching HP was performed with a stock solution of 50 mg/L catalase (Sigma, St. Louis, MO), which was prepared by dissolving catalase in 50 mM potassium phosphate buffer (pH 7.0) (Wagner et al., 2002). Residual measurement before and after quenching was performed using the ABTS-HRP method described above. Catalase solutions were stored at 4°C in 1 mL aliquots and tested daily for sterility. Catalase is an antioxidant and used to scavenge unreacted HP molecules from further affecting bacterial cells (Cho et al., 2006a; Wagner et al., 2002).

Figure 3.11: ABTS-HRP method to measure concentration of HP in solution diluted with phosphate buffer or wastewater

3.2.4 Peracetic acid

VigorOx WWTII PAA solution containing 15% PAA and 23% HP with a specific gravity of 1.16 g/mL at 20°C, (FMC Corporation, Philadelphia, PA) was used throughout testing. Quenching was performed with a 0.02% (w/v) solution of sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3$) (Fisher Scientific, New Jersey NY) with a 2 minute reaction time followed by 50 mg/L catalase dissolved in 50 mM potassium phosphate buffer (pH 7) (Sigma, St. Louis MO). Catalase was used to quench any residual concentrations of HP (Cho et al., 2006a; Wagner et al., 2002). The sterility of the catalase solution was assured daily by testing 10 μL and 100 μL of the catalase stock solution by membrane filtration on EC Medium with MUG (Becton Dickinson and Company, Sparks, MD, USA), see Section 3.4 for incubation times and conditions. The effectiveness of the quenching protocol was

assured by using the ABTS-HRP colorimetric method described in Section 3.2.3. Alternatively, the PAA concentration and quenching was confirmed by using Hach Method 10101. The change of the residual concentration of PAA in wastewater with time using the latter method can be seen in Figure 3.12.

Figure 3.12: Residual concentration of PAA with time with starting concentrations of 1.5, 2, and 3 mg/L

3.3 Experimental matrix

Secondary effluent wastewater was collected weekly from the Robert O. Pickard Environmental Center (Ottawa, ON) in sterile 4-L Nalgene containers and kept at 4°C until used. The treatment plant has a conventional treatment train serving approximately 883,000 residents with average daily flows of 390,000 m³/day. Wastewater was spiked with an overnight culture of *E. coli* at a ratio of 2:100 to achieve

consistent starting concentrations of 7 Log CFU/mL. *B. subtilis* suspensions were spiked into autoclaved (at 121°C for 15 minutes) secondary wastewater at a ratio of 2:100 achieving starting concentration of 5 Log CFU/mL.

3.4 Bacteria species

Throughout the study, a number of bacteria species were used to investigate disinfection performance of the various disinfection treatments. Below are the source and growing conditions used to make the cultures of the selected strains.

3.4.1 Escherichia coli

E. coli K-12 strain 3000-141 (ATCC® 19853, Cedarlane Laboratories, ON) was used throughout experimentation. Frozen culture (10^9 CFU/mL) was first revived overnight at 37°C in Bacto™ Tryptic Soy Broth Soybean-Casein Digest (Becton, Dickinson and Company, Franklin Lakes NJ) in a ratio of 1:100 after which suspensions were washed 3 times in the centrifuge for 5 minutes at 10,000xg with phosphate buffered solution (PBS) (Standard Methods, 2005). Experimental suspensions were created by adding 20 mL of the washed *E. coli* suspension to 1 L of secondary effluent wastewater for initial colony counts of approximately 10^7 CFU/mL.

3.4.2 Coliform bacteria

Coliform bacteria were cultured directly from wastewater samples.

3.4.3 Clostridium perfringens

C. perfringens is a pathogenic bacterium and has a biosafety level 2 classification that requires special safety considerations. During handling and experimentations, the

experimenter wore an N95 mask to prevent inhalation of aerosols and worked under biosafety hood as much as possible. Laboratory safety goggles and latex-free gloves were also worn at all times and disposed of in appropriate containers. All waste, including experimental solutions, were autoclaved after use at 121°C for a minimum of 15 minutes or exposed to a 2% aqueous glutaraldehyde for 10 hours within 3 hours of exposure before disposal. Surfaces were cleaned before and after experiments with commercial-grade accelerated HP solution, Virox. *C. perfringens* were cultured directly from wastewater samples.

3.4.4 Bacillus subtilis

B. subtilis (ATCC® 19659 Cedarlane Laboratories, ON) were revived overnight at 37°C ± 2°C by streaking frozen stock culture on TSB with 1.5% Laboratory Grade Agar (Bishop, Burlington, ON) The mixture of tryptic soy broth and agar will be referred to tryptic soy agar or TSA. A spore stock solution was made by streaking overnight culture onto TSA for 26 days at 37°C ± 2°C after which the formation of spores was confirmed by phase contrast microscopy. Colonies were washed with PBS and heated to 70°C for 20 minutes to inactivate vegetative cells. The heat-treated suspension was washed 8 times with deionized water by centrifuging at 10,000xg for 10 minutes to remove vegetative cells (Kornberg and Spudich, 1969). Malachite green was used to stain and enumerate spores, see Figure 3.13. The final suspension had a cell concentration of 3.7×10^8 CFU/mL *B. subtilis* containing 65% spores and stored at 4°C for up to four weeks. *B. subtilis* are not naturally found in wastewater samples but are often used to study the effect of

disinfection treatment on spore forming bacteria. Experiments with *B. subtilis* were performed by spiking autoclaved wastewater.

Figure 3.13: *B. subtilis* stained with malachite green imaged with Nikon Eclipse TE oil immersion light microscope at 100x magnification (a) mixed vegetative and spore suspension (b) mixed vegetative and spore suspension after washing and heat treatments, this suspension was used in experiments.

3.4.5 *Pseudomonas aeruginosa*

Two strains of *P. aeruginosa* were used in this study, *P. aeruginosa* ATCC® 19853, and *P. aeruginosa* PAO1H2O. Stock solutions of both strains of *P. aeruginosa* were revived overnight at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and washed 3X with PBS before use.

P. aeruginosa PAO1H2O was isolated from wastewater. To do so, a serial dilution of wastewater was plated on Pseudomonas Isolation Agar (PIA) (Fluka Analytical, 172208) and incubated for 24 hours at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Positive *P. aeruginosa* colonies were identified by positive fluorescence under black light and the presence of pyoverdine (green) pigmentation. Further confirmation was performed with 16s RNA-PCR amplification, Table 3.2 for primer selection. To complete the 16s RNA-PCR amplification, genomic

DNA was extracted with MoBio (Carlsbad, CA, USA). Each PCR reaction contained 30 ng genomic DNA measured with the Qubit 3.0 fluorometer (Invitrogen by Life Technologies, New Jersey NY), 12.5 μ L SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA), 200 nM primers (obtained from a primer stock of 10 μ M) and filled to 25 μ L with Sigma water (Sigma-Aldrich, Saint Louis, MO). Cycling parameters included an initial denaturation at 95.0°C for 5 min, followed by 40 cycles of 95.0°C for 10s, 60.0°C for 20s and 72.0°C for 15s, see Figure 3.14-a. A melt curve was also performed for quality control with increasing temperatures from 65.0°C to 95°C in 0.5°C increments for 5s, see Figure 3.14-b. All reactions were performed in triplicate and analyzed with the Bio-Rad CFX96-C1000 Real-Time PCR system and the Bio-Rad CFX Manager 3.1. PCR amplicon was cleaned with QIA quick PCR Purification Kit (QIAGEN Sciences Maryland, USA). Gel electrophoresis used to confirmed that the isolated DNA was of the correct size (i.e. the correct number of base pairs) and was performed using 2.5% agarose gel at 90 volts for 70 min and stained with 0.004% (w/v) ethidium bromide in 1X TAE for 30 minutes to demonstrate the size and quality of the PCR fragment, see Figure 3.15. High-throughput DNA sequencing was performed with the Applied Biosystems 3730 DNA Analyzer by StemCore Laboratories (Ottawa, ON). The sequence was then inputted into BLAST (blast.ncbi.nlm.nih.gov) where *P. aeruginosa* PAO1H2O was confirmed with an E-value range of 1×10^{-39} - 2×10^{-41} .

Table 3.2: 16s qPCR primers used in the study

Genetic Target	Primer	Sequence (5' → 3')	PCR Product Size (bp)	Reference
16s	341	CCTACGGGAGGCAGCAG	193	Muyzer, de Waal, and Uitterlinden, 1993
	534	ATTACCGCGGCTGCTGG

Figure 3.14: (a) 16s RNA-PCR amplification curve of *P. aeruginosa* isolated from wastewater $C_q = 12.19$ (blue circles) and non-template control $C_q = 24.49$ (red crosses) and (b) associated melt curve of 16s RNA-PCR amplification of *P. aeruginosa* isolated from wastewater (blue circles) and non-template control (red crosses)

Figure 3.15: 2.5% agarose gel stained with ethidium bromide Lane 1: 50 bp ladder; Lane 2: intentionally left empty; Lane 3: 16s amplicon from *P. aeruginosa* isolated from wastewater, image was taken with Canon 60D EOS with EF-S 18-55mm f/3.5-3.6 IS lens with Kenko YA3(orange) 50-56, 58mm and a UVIR filter

3.5 Culture conditions and enumeration of bacteria

The membrane filtration procedure outlined in Protocol 9222 B of *Standard Methods* was used to enumerate each bacteria type using membrane filtration units by Advantec (Japan) along with 0.45 μm , 47 mm S-Pak™ membrane filter by Millipore water (Billerica, MA, USA), unless otherwise specified (Standard Methods, 2005). Filtration was vacuum assisted with a Vacuum Pressure Pump by Barnant Co, model number 400-1901 (Barrington, IL, USA). Each dilution was performed with a minimum of three replicates, which were diluted in room-temperature autoclave-sterilized phosphate buffered solution (PBS) (Standard Methods, 2005). Once the filtration was complete, the membrane filter was removed from the platform with sterile forceps and placed on an agar-filled 47mm Petri dish, which was then incubated for a specified amount of temperature and time, depending the organism being cultured. Agar selection was also organism specific. Following the required incubation period, plates were removed from

the incubator and colonies with the necessary morphology were counted if between 1 and 200 colonies were present. Details of the agars, growth conditions as well as colony morphology can be seen in Table 3.3.

Table 3.3: Bacterial strains, growing conditions, and colony morphology

Bacteria Species	Agar	Incubation Criteria	Enumeration & Morphology
<i>E. coli</i>	Bacto™ EC Medium with MUG by BD Canada (Mississauga, ON) + 1.5% (w/v) Agar Laboratory Grade ARG003.500 by Bishop (Burlington, ON)	Aerobic incubation 24 ± 2 hours at 37°C	Colonies fluoresce under black light
Coliform Bacteria	M-Endo agar–Difco™ LES by BD Biosciences (Mississauga, ON)	Aerobic incubation 24 ± 2 hours at 37°C	Metallic colonies
<i>C. perfringens</i>	CP Chromo Select Agar + M-CP selective Supplement I by Fluka Analytical (St. Louis, MO)	Anaerobic* 24 ± 2 hours at 44°C + aerobic 2 ± 1 hours at 44°C	Green colonies
<i>B. subtilis</i>	Bacto™ Tryptic Soy Broth Soybean-Casein Digest Medium by BD Bioscience (Sparks MD) + 1.5% (w/v) Agar Laboratory Grade ARG003.500 by Bishop (Burlington, ON)	Aerobic incubation 24 ± 2 hours at 37°C	Colorless colonies with wrinkled edges
<i>P. aeruginosa</i>	Pseudomonas Isolation Agar (PIA) by Fluka Analytical (St. Louis, MO)	Aerobic incubation 24 ± 2 hours at 37°C	Colonies fluoresce under black light

*Anaerobic conditions were achieved by placing plates in anaerobic jars used with OXOID AnaeroGen anaerobic gas packs. Anaerobic conditions were assured with OXOID Anaerobic Indicator (Thermos Scientific, Eugene Oregon USA)

3.6 Wastewater quality parameters

Absorbance scans and single wavelength absorbance measurements were performed with a UV-visible spectrophotometer (Varian Model Cary 100) that was zeroed with ultrapure water with 18.2 MΩ.cm at 25°C (Millipore Direct-q UV 3) (Millipore, Etobicoke, ON). Hach colorimetric tests were used to determine the concentration of wastewater characteristics with the DR 2800 Portable Spectrophotometer. Chemical oxygen demand (COD) was determined using COD Digestion Vials High Range 20-1500 mg/L and Hach method 8000. Ammonia was determined using the AmVer™ Salicylate Test 'N Tube™ Method with Hach method 10031, and nitrite with the Nitrite TNTplus TNT839. Nitrate was measured with TNT NitraVer® X following Hach method 10020. Total phosphorous with the PhosVer®3 Acid Persulfate Digestion Method 8190, and sulfate with the SulfaVer4 with Hach Method 8051. Turbidity measurements were performed with the Hach 2100AN turbidimeter. Calibration of the turbidimeter was conducted using the StableCal® Calibration.

3.7 Propidium monoazide protocol, and DNA extraction and quantification

PMA stock solutions were created by dissolving 5 µL of 20 mM Propidium monoazide (PMA) (Biotium, Hayward, CA) in 20% dimethyl sulfoxide (DSMO) (Caledon Laboratory Chemicals, Georgetown, ON) and stored in at 4°C and shielded from light in a sterile dark glass vial until use. A volume of 5 µL of the PMA stock solution was added to 1 mL of treated sample, mixed gently with a vortex mixer for 5 seconds and incubated in the dark, at room temperature, for 10 min. The mixtures of PMA and samples were then

exposed to a 150-watt halogen bulb for 10 minutes on ice and gently mixed by hand every two minutes to allow for the PMA to bind to the extracted DNA. It was observed that the halogen light supplied heat to the samples and as such samples were placed on ice during exposure. Following, the mixtures were centrifuged for 3 minutes at 10,000xg and a pellet was formed in each tube. The supernatant of was discarded. The pellet were then processed with PowerSoil DNA Isolation Kit (Mobio, Carlsbad CA, USA) to extract genomic DNA according to manufacturer's instructions with the exception that the bead-beating and incubation periods were increased to 10 minutes. DNA extracts were quantified using the Qubit 3.0 Fluorometer (Life Technologies, Eugene, OR). Processed samples were stored at -20°C until use for qPC (see Section 3.8) following manufacturer's instructions. Figure 3.16 demonstrates a characteristic data obtained after detailing the quantity of DNA obtained before (non-hashed columns) and after (hashed columns) the PMA treatment. Figure 3.16 shows results after disinfection treatment with PAA. Comparing the columns, between 51% and 95% less DNA was extracted after the PMA treatment, indicating the efficiency of the PMA treatment. Further, higher reductions of the quantity of DNA extracted after PMA treatment were seen with increased concentrations of PAA.

Figure 3.16: Representative graph showing the quantity of DNA [ng/μL] measured with the Qubit Fluorometer after wastewater was disinfected with PAA at concentrations of 0.52, 1.16, 2.32 and 4.64 mg/L

3.8 Real-time qPCR

The qPCR reactions were performed with 25 μL containing: 5μL template, 2.5μL 10xPCR Buffer, 1.25 μL 50mM MgCl, 0.5 μL 10 mM dNTPs, 1.25 μL 5% DMSO, 0.625 μL of 4 μM stock of forward primer, 0.625 μL of 4 μM stock of reverse primer, 1 μL 10 μg/μL BSA, 20X EvaGreen, 0.5μL Native taq and 10.5 μL Sigma Water. Primers coding *uidA* gene, which has been shown to appear in 97.7 % of *E. coli* isolates from water samples (Martins et al., 1993), and appears only once per genome (Taskin et al., 2011) were selected, see Table 3.4 for details of the primers selected. Cycling parameters included denaturation at 95.0°C for 2 min, followed by 40 cycles of 95.0°C for 10 s, 61.4°C for 20 s and 72.0°C for 15 s. After every qPCR run, a melt curve was performed for quality control purposes with increasing temperatures from 65.0°C to 95°C in 0.2°C increments,

each increment lasting 5s. All reactions were performed in triplicate and analyzed with the Bio-Rad CFX96-C1000 Real-Time PCR system and the Bio-Rad CFX Manager 3.1. Each qPCR plate included negative and positive controls as well as a standard curve created by a serial dilution of *E. coli*-spiked wastewater. Relative quantification analysis was used throughout to report results in calculated CFU equivalents (CCE). Figure 3.17 shows representative serial dilution of genomic DNA extracted from pure culture *E. coli*, the corresponding melt curve as well as the resulting standard curve for converting Cq to CCE.

Table 3.4: uidA qPCR primers used in the study

Genetic Target	Primer	Sequence (5' → 3')	PCR Product Size (bp)	Reference
<i>uidA</i> gene	UAL 1939b	ATGGAATTTGCGCCGATTTTGC	187	(Heijnen and Medema, 2006a)
	UAL 2105b	ATTGTTTGCCTCCCTGCTGC

Figure 3.17: Representative graphs taken from HP experiments of (a) *uidA* amplification curve of serial dilution of *E. coli* spiked in wastewater, showing linear range $C_q = 15.31, 18.39, 22.25, 25.37, 29.02$ (blue line) with positive control *E. coli* $C_q = 12.16$ (green crosses) and no template control $C_q = 30.46$ (red circles); (b) the associated melt curve of (blue line), positive control (green crosses) and no template control (red circles) with a melt temperature = 84.40°C ; (c) standard curve used for converting C_q to CCE/mL

3.9 Live/dead viability stain

The LIVE/DEAD BacLight® Bacterial Viability Kit L13152 (Molecular Probes, Eugene OR) was used to stain and visualize viable and non-viable cells. The kit contains two nucleic acid stains. SYTO-9 is a green fluorescing dye that labels all nucleic acid. Propidium iodide is a red fluorescing dye that penetrates cells with damaged membrane labeling these cells red. When used together, green fluorescing cells indicate viable cells (as measured by a healthy cell membrane) and a red fluorescing cell indicates non-viable cells. Dyes from the kit were hydrated in a common 5 mL of deionized water and stored at 4°C in a sterilized dark glass container for up to one month. To perform staining,

samples were mixed in equal volume amounts with hydrated dye mixture, gently vortexed for 5 seconds and incubated in the dark at room temperature for 10 minutes. Slides imaged with the Eclipse TE (Nikon, Montreal QC) microscope equipped with Intensilight C-HGFI fluorescent illuminator (Nikon, Montreal QC) and GFP-1, Cy3, GFPHQ filters (Nikon, Montreal QC). To obtain a representative image slides visually scanned and preliminary cell counts were taken for a minimum of three fields of view. A picture of a representative field of view was taken with the Q Imaging Retina Exi Fast 1394 (Nikon, Montreal QC) camera and cell counts and image processing completed with NIS-Elements software (Nikon, Montreal QC). A representative field of view of the live/dead images of an initial sample of wastewater are shown in Figure 3.18.

Figure 3.18: Representative live and dead fluorescent image from wastewater sample containing 10^7 CFU/mL E. coli

3.10 Minimal media

Minimal media solutions were prepared by adding 20 mL 5X M9 salts (Sigma-Aldrich, ST. Louis, MO), 0.2 mL autoclaved 1M $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ (BioShop, Burlington, ON), and 0.01 mL autoclaved 1M calcium chloride (CaCl_2) (Fisher Scientific, Rochester, NY) to 75 mL of autoclaved deionized water and diluted to 96 mL (Sambrook and Russell, 2001). A volume of 9.6 mL was aliquoted into sterilized 15 mL conical centrifuge tubes to which either, no food (negative control), 0.2 mL filtered-sterilized solution of 20% glucose (positive control) (Sigma-Aldrich, ST. Louis, MO) or 0.2 mL filtered-sterilized 20% acetic acid (Sigma-Aldrich, St. Louis, M) were added. Solutions were spiked with cultures of *E. coli* or *P. aeruginosa* to achieve an initial bacterial concentration of approximately 10^9 CFU/mL. All minimal media solutions were incubated for 24 hours at $37^\circ\text{C} \pm 2^\circ\text{C}$. Bacteria concentrations were evaluated using the culture methods previously described Sections 3.4 and 3.5.

3.11 Statistical analysis

One-way analysis of variance (ANOVA) was performed to determine if a significant difference exists between the means of two or more samples. The alternative hypothesis, which means were different, was accepted if the *p*-value was less than the significance level (α) of 0.05. If accepted, the Tukey test was used posthoc to separate the means further, using the same significance level (Currell and Dowman, 2005). Statistical analysis was performed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

Unless otherwise stated, data represents the average of three replicates and the error bars represent the standard deviation from the mean.

3.13 References

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Chapter 4

4 Survival of viable but nonculturable *Escherichia coli* in wastewater following disinfection with sodium hypochlorite, hydrogen peroxide, peracetic acid, ferrate(VI), and ultraviolet light

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Abstract

Wastewater disinfection aims to reduce the level of pathogenic bacteria and viruses before treated wastewater is released into the environment. Culture methods are often part of the routine analysis for assessing the microbial quality of wastewater. However, growing evidence indicates that culture methods alone do not provide a complete picture. After disinfection, a portion of bacteria found in wastewater can enter a viable but nonculturable (VBNC) state where they can no longer be cultured but may retain metabolic activity. This study investigated the fate of *E. coli* in wastewater following disinfection, with a number of treatments including sodium hypochlorite (NaClO), hydrogen peroxide (HP), peracetic acid (PAA), ferrate(VI), and ultraviolet light (UV). A molecular method combining the use of propidium monoazide (PMA) and real-time quantitative polymerase chain reaction (qPCR) was used to quantify DNA of viable cells. Results were compared and contrasted to traditional culture methods as well as to images taken using a live/dead viability stain. All disinfectants were able to reduce culturable bacteria to below standard compliance levels of 200 CFU/mL (Ontario,

Canada) as measured by routine culture methods. However, the PMA-qPCR molecular method showed that a significant portion of cells remained viable but were in a nonculturable state following disinfection with NaClO, HP, and PAA and ferrate(VI). Ferrate(VI) was the most effective at reducing VBNC bacteria. Disinfection with PAA and NaClO showed similar levels of bacteria in the VBNC state. The PMA-qPCR method was not able to detect VBNC cells after UV disinfection since UV does not cause significant membrane damage. Results of the study indicate that a large number of cells survive chemical disinfection in a VBNC state.

Keywords: Wastewater, disinfection, qPCR, PMA-qPCR, VBNC, *E. coli*, chlorine, sodium hypochlorite, UV, ferrate(VI), peracetic acid, hydrogen peroxide

4.1 Introduction

The purpose of wastewater disinfection is to reduce the level of enteric microorganisms before treated wastewater is released into the environment. Culturing indicator organisms on selective media has long been the operating standard to assess the microbial quality of effluent wastewater (World Health Organization and Organisation for Economic Co-operation and Development, 2003) despite the fact that the absence of the colony growth does preclude the presence of pathogenic microorganisms, more resistant microorganisms, or the presence of viruses and protozoa. In addition, bacteria may lose culturability but retain some measurable metabolic activity in physiological state termed VBNC (Calgua et al., 2014; Oliver, 2005; Oliver et al., 2005; Ramamurthy et

al., 2014; Xu et al., 1982). The VBNC state was first mentioned in 1982 when researchers observed that *E. coli* and *Vibrio cholera* demonstrated a loss of culturability after being incubated in saltwater, but maintained viable characteristics such as a positive substrate-response using direct viable count (DVC) (Xu et al., 1982). Since then, over 60 bacterial strains have been shown to exist in a VBNC-state (Oliver, 2010, 2005), the induction of which is considered to be a survival response to stressors such as nutrient starvation (K. L. Cook and Bolster, 2007), exposure to low temperatures (Maalej et al., 2004; Wong and Wang, 2004), low oxygen conditions (Kana et al., 2008), and exposure to white light as well as sunlight (Besnard et al., 2002; Gourmelon et al., 1994).

Research is still investigating the VBNC-state. While some microorganisms have been shown to continue to express virulence genes (Oliver et al., 1995b; Ramamurthy et al., 2014), this is not the case with in other circumstances (Sun et al., 2008). In addition, factors that trigger bacteria to exit or resuscitate from the VBNC-state have also been difficult to define (Barcina and Arana, 2009; Coyle and Ormsbee, 2008; Ramamurthy et al., 2014; Sachidanandham and Gin, 2009). The ability of a VBNC-bacteria to cause infectivity has also yet to be confirmed (Ramamurthy et al., 2014).

For the wastewater treatment plant (WWTP), oxidative stress due to chemical disinfection has been shown to induce the VBNC-state (Noor et al., 2009; Oliver et al., 2005; Servais et al., 2009). For example, a study that looked at the different states of *E. coli* and *Salmonella typhimurium* after exposure to chlorine found that 4 Log cells/mL of the initial 6 Log cells/mL survived in a VBNC state as determined by DVC (Oliver et al.,

2005). The concern is that VBNC bacteria may resuscitate and regain full metabolic activity and virulence downstream. However, mixing treated wastewater with low-nutrient receiving water has not been shown to favor resuscitation. When introduced into a low-nutrient environment, such as drinking water, Byrd et al. (1991) observed the loss of culturability of three gram-positive bacteria (*M. flavus*, *S. feacalis*, *B. subtilis*) and five gram-negative bacteria (*A. Tumefaciens*, *E. aerogenes* and *K. pneumoniae*, *Pseudomonas sp.* strain 719 and *P. fluorescens* L-2). Bacteria were shown to remain in this state for periods extending beyond 95 days or the duration of the experiment (Byrd et al., 1991). In a separate study, quantities of culturable and viable *E. coli* varied depending on the distance from the wastewater effluent mixing points. Samples taken near the wastewater outlets had viable and culturable bacteria while samples taken further downstream or taken from shallow bodies of water had a higher number of VBNC bacteria. The increase in VBNC bacteria was due to the lack of nutrients in the natural water sources as well as from stress from solar radiation (Servais et al., 2009).

The most commonly used methods to detect VBNC bacteria are the DVC, the live/dead viability staining, and reverse transcriptase-polymerase chain reaction (RT-PCR), which can all have a limitation in natural and wastewater samples (Oliver, 2010). A recent molecular method combining the use of DNA-intercalating dye propidium monoazide (PMA) and quantitative polymerase chain reaction (qPCR) has been developed for dilute and complex matrices such as drinking water (Zhang et al., 2015), wastewater (Bae and Wuertz, 2009; Kibbee and Örmeci, 2017; Varma et al., 2009) and sludge samples (van Frankenhuyzen et al., 2013, 2011).

The purpose of this research was to examine the culturable and VBNC states of indicator organism *E. coli* in a wastewater matrix after a number of disinfection treatments including NaClO, peracetic acid (PAA), hydrogen peroxide (HP), ferrate(VI), and ultraviolet light (UV) to determine the quantity of VBNC-bacteria remaining after disinfection. In addition, this research aimed to evaluate and compare disinfectants ability to reduce VBNC-bacteria. To complete the research, disinfected wastewater was analyzed in parallel with conventional culture techniques, qPCR, PMA-qPCR, and the live/dead viability staining. Typical disinfection concentrations were used to assess the current state and level of VBNC bacteria escaping detection at WWTP after each disinfection treatment.

4.2 Materials and methods

4.2.1 Bacterial strains

E. coli strain ATCC®19853 (Cedarlane Laboratories, Burlington, ON, Canada) was used throughout experimentation. Frozen culture (10^9 CFU/mL) was first revived overnight at 37°C in Bacto™ Tryptic Soy Broth Soybean-Casein Digest (BD, Mississauga, ON, Canada) in a ratio of 1:100 after which suspensions were washed a total 3 times centrifuging suspensions for 5 minutes at 10,000xg discarding the supernatant and resuspending in phosphate buffered solution (PBS) (Eaton et al., 2005). Experimental suspensions were created by adding 20 mL of the washed *E. coli* suspension to 1 L of secondary effluent wastewater for initial colony counts of approximately 10^7 CFU/mL.

4.2.2 Effluent Wastewater

Secondary effluent wastewater was used as the experimental matrix throughout experimentation. Samples were collected from a local treatment plant using conventional treatment (primary sedimentation, activated sludge, secondary sedimentation and chemical nutrient removal) serving approximately 883,000 residents with average daily flows of 390,000 m³/day. Samples were taken from the secondary clarifier following secondary sedimentation and transported directly to the lab. Samples were collected weekly and kept at 4°C until use. The use of wastewater as the experimental matrix allowed for a realistic view of the fate of bacteria after treatments. After spiking, starting concentrations of *E. coli* were approximately 1-log higher than what was naturally found in wastewater and allowed for consistent experimental starting conditions.

4.2.3 Chemical treatments

Jar testers were used to perform chemical disinfection experiments. Samples were placed in 1-L beakers covered in foil to shield samples from light. Mixing was provided at a rate of 70 rpm.

4.2.3.1 Sodium hypochlorite

Chlorine experiments were performed with 5.25% sodium hypochlorite (NaOCl) without pH adjustments. Total and free chlorine were measured using HACH methods 8167 with N, N-Diethyl-p-Phenylenediamine (DPD) Total Chlorine Reagent and DPD Free Chlorine Reagent (Hach Permachem Reagents, Mississauga ON) in conjunction with the Hach

DR2800 Spectrophotometer. Quenching was performed with a 0.02% (w/v) solution of sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) (Fisher Scientific, New Jersey NY).

4.2.3.2 Hydrogen peroxide

Household solutions of 3% HP were used without further dilution throughout and quenched with 50 mg/L catalase (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 50 mM potassium phosphate buffer (pH 7.0). Catalase solutions were stored at 4°C in 1 mL aliquots and tested daily for sterility. Catalase is an antioxidant and used to scavenge HP molecules (Cho et al., 2006a; Wagner et al., 2002).

4.2.3.3 Peracetic acid

VigorOx WWTII PAA solution containing 15% PAA and 23% HP with a specific gravity of 1.16 g/mL at 20°C, (FMC Corporation, Philadelphia, PA, USA) was used throughout. Quenching was performed with sodium thiosulfate followed by catalase (Falsanisi et al., 2008; Wagner et al., 2002).

4.2.3.4 Ferrate(VI)

Stock solutions of ferrate(VI) were created by dissolving of 1 g/L of potassium ferrate(VI) salts (K_2FeO_4) (>90%, Sigma-Aldrich, St. Louis, MO, USA) in ultrapure water dispensed from the Millipore Direct-q UV 3 (Millipore Sigma, Etobicoke, ON, Canada). Stock solutions had a pH of 11 and were stored in sterilized dark glass bottles at 4°C until used. The concentration of the ferrate(VI) ion, FeO_4^{2-} , was verified using the direct colorimetric method (Luo et al., 2011). Stock solutions were discarded after three hours due to an observed 10% loss of ferrate(VI) ion concentration. Experiments were performed without pH adjustments and maintained a contact time of 30 minutes.

Quenching was carried out with 0.02% (w/v) solution of sodium thiosulfate. Grab samples were taken directly from jar testers with no additional settling time provided. Throughout the study, the notation of ferrate(VI) or Fe(VI) was used to distinguish from ferrate(V).

4.2.4 Ultraviolet light

A bench-scale collimated beam apparatus was used for all UV experiments. Experimental procedures and germicidal fluence calculations followed those for suspension depth of less than 2 cm (Bolton and Linden, 2003). Sample volumes of 150 mL were placed in tall-walled sterile glass Petri dishes and placed on a mixing plate that also served as the irradiation stage and allowed continuous mixing within the UV reactor. A set of four low-pressure mercury lamps (Phillips UV-C germicidal lamps) provided nearly-monochromatic UV light at 253.7 nm, and two apertures placed below the lamps assured collimation of the beam. The inside of the apparatus was painted with a matt black paint to ensure little to no reflectivity from surfaces. The absorption coefficient at 254 nm was measured before every experiment with the UV-Visible spectrophotometer (Varian Model Cary 100BIO) using a 1-cm quartz cuvette. Irradiance from the lamps was recorded with the International Light NIS Traceable Radiometer/Photometer (Model IL 1399A).

4.2.5 Culture enumeration

Membrane filtration procedure outlined in Protocol 9222 B of *Standard Methods* was used to enumerate *E. coli* before and after treatment (Eaton et al., 2005). Each dilution was performed with a minimum of three replicates diluted with room-temperature

sterilized phosphate buffered solution (PBS) (Eaton et al., 2005). Bacto™ EC Medium with MUG (BD, Sparks, MD, USA) was used throughout with incubation taking place at 35°C for 24 ± 2 hours. Positive *E. coli* colonies fluoresced under black light.

4.2.6 Propidium monoazide protocol, and DNA extraction and quantification

A stock solution of 20 nM of propidium monoazide (PMA) (Biotium, Hayward, CA, USA) was made PMA in 20% dimethyl sulfoxide (DSMO) (99.9%, Caledon Laboratory Chemicals, Georgetown, ON, Canada) and stored in at 4°C sterile dark glass vial to shield the mixture from light until use. A volume of 5 µL of the PMA stock solution was added to 1 mL of treated sample, mixed gently with a vortex mixer for 5 seconds and incubated in the dark at room temperature for 10 min. PMA-sample mixtures were then exposed to a 150-watt halogen bulb at a distance of 20 cm for 10 minutes on ice and gently mixed by hand every two minutes. Following, solutions were centrifuged for 3 minutes at 10,000xg to form a pellet and the supernatant discarded. Samples were then processed with PowerSoil DNA Isolation Kit (Mobio, Carlsbad CA, USA) to extract genomic DNA according to manufacturer's instructions with the exception that the bead-beating and incubation periods were increased to 10 minutes. DNA extracts were quantified using the Qubit 3.0 Fluorometer (Life Technologies, Eugene, OR, USA) and stored at -20°C until used for qPCR analysis. Figure 4.1 demonstrates a characteristic data obtained detailing the quantity of DNA obtained before (non-hashed columns) and after (hashed columns) PMA treatment of a 1 mL sample of wastewater treated with the same PAA treatment. Comparing the columns, between 51% and 95% less genomic DNA was extracted after treatment with PMA, indicating the efficiency of the PMA

treatment. Further, higher reductions of the quantity of DNA extracted after PMA treatment were seen with increased concentrations of PAA.

Figure 4.1: Representative graph showing the quantity of DNA [ng/μL] measured with the Qubit Fluorometer after wastewater was disinfected with PAA at concentrations of 0.52, 1.16, 2.32 and 4.64 mg/L

4.2.7 Real-time qPCR

Each qPCR reaction was performed with 25 μL reaction volume that contained: 5 μL DNA template, 2.5 μL 10xPCR Buffer (Invitrogen, Carlsbad, CA, USA), 1.25 μL 50mM MgCl (Invitrogen, Carlsbad, CA, USA), 0.5 μL 10 mM dNTPs (Invitrogen, Carlsbad, CA, USA), 1.25 μL 5% DMSO min (99.9%, Caledon Laboratory Chemicals, Georgetown, ON, Canada), 0.625 μL of 4 μM stock of forward primer, 0.625 μL of 4 μM stock of reverse primer, 1 μL 10 μg/μL BSA, 20X EvaGreen (Biotium Inc., Fremont, CA, USA), 0.5μL DNA Taq Polymerase (Native, Invitrogen, Carlsbad, CA, USA) and filled to 25μL with DNA-free water (Sigma-Aldrich, St. Louis, MO, USA). Primers coding *uidA* gene, which has been

shown to appear in 97.7 % of *E. coli* isolates from water samples (Martins et al., 1993), and appears only once per genome (Taskin et al., 2011) were selected, see Table 4.1 for details. Cycling parameters included denaturation at 95.0°C for 2 min, followed by 40 cycles of 95.0°C for 10 seconds, 61.4°C for 20 seconds and 72.0°C for 15 seconds. Each qPCR plate included a serial dilution of pure culture *E. coli* diluted in wastewater (indicated by the blue lines in Figure 4.2-a) as well as a negative control where no DNA was added to the reaction volume (indicated by red circles in Figure 4.2-a) and a positive control where DNA extracted from pure culture *E. coli* was added (indicated by green crosses in Figure 4.2-a). Figure 4.2-a was used to create a standard curve, shown in Figure 4.2-c, following the absolute quantification method of analysis to convert Cq values reported by the qPCR to calculated CFU equivalents (CCE) (Bio-Rad Laboratories, 2006). After every qPCR run, a melt curve was performed to assure specific amplification of the target region of DNA as shown by a single peak at 84.40°C. The curve was performed by increasing the temperature from 65.0°C to 95°C in 0.2°C increments, each increment lasting 5 seconds, see Figure 4.2-b. All reactions were performed in triplicate and analyzed with the Bio-Rad CFX96-C1000 Real-Time PCR system and the Bio-Rad CFX Manager 3.1.

Table 4.1: qPCR primers used in the study

Genetic Target	Primer	Sequence (5' → 3')	PCR Product Size (bp)	Reference
<i>uidA</i> gene	UAL 1939b	ATGGAATTTGCGCGATTTTGC	187	(Heijnen and Medema, 2006a)
	UAL 2105b	ATTGTTTGCCTCCCTGCTGC

Figure 4.2: Representative graphs taken from HP experiments of (a) *uidA* amplification curve of serial dilution of *E. coli* spiked in wastewater, showing linear range $C_q = 15.31, 18.39, 22.25, 25.37, 29.02$ (blue line) with positive control *E. coli* $C_q = 12.16$ (green crosses) and no template control $C_q = 30.46$ (red circles); (b) the associated melt curve of (blue line), positive control (green crosses) and no template control (red circles) with a melt temperature = 84.40°C ; (c) standard curve used for converting C_q to CCE/mL

4.2.8 Live/dead viability stain

The LIVE/DEAD BacLight® Bacterial Viability Kit L13152 (Molecular Probes, Eugene OR) was used to stain and visualize viable and non-viable cells. The kit contains two nucleic acid stains. SYTO-9 is a green fluorescing dye that labels all nucleic acid. Propidium iodide is a red fluorescing dye that penetrates cells with damaged membrane labeling these cells red. When used together, green fluorescing cells indicate viable cells (as measured by a healthy cell membrane) and a red fluorescing cell indicates non-viable cells. Dyes from the kit were hydrated in a common 5 mL of deionized water and stored at 4°C in a sterilized dark glass container for up to one month. To perform the staining, samples were mixed with equal volume amounts with hydrated dye mixture, gently vortexed for 5 seconds and incubated in the dark at room temperature for 10 minutes. Slides imaged with the Eclipse TE (Nikon, Montreal QC) microscope equipped with Intensilight C-HGFI fluorescent illuminator (Nikon, Montreal QC) and GFP-1, Cy3, GFPHQ filters (Nikon, Montreal QC). To obtain a representative image slides visually scanned and preliminary cell counts were taken for a minimum of three fields of view. A picture of a representative field of view was taken with the Q Imaging Retina Exi Fast 1394 (Nikon, Montreal QC) camera and cell counts and image processing completed with NIS-Elements software (Nikon, Montreal QC). See Figure 4.3 for a representative view live (green) and dead (red) bacteria of initial wastewater sample spiked with *E. coli*.

Figure 4.3: Representative live and dead fluorescent image from wastewater sample containing 10^7 CFU/mL *E. coli*

4.3 Results and discussion

4.3.1 Sodium hypochlorite

Concentrations of 1.0 to 3.8 mg/L as Cl_2 of NaClO with contact times of 30 minutes were evaluated. Wastewater collected throughout the study came from a treatment plant that maintained compliance requirements using NaClO year round at concentrations between 2 and 2.5 mg/L Cl_2 . Figure 4.4 compares results from culture, qPCR, and PMA-qPCR methods. Results of the culture method as shown in the first series of columns in Figure 4.4 demonstrate a gradual decrease of culturable *E. coli* from 7.59 to 0.38 Log CFU/mL increasing concentrations of chlorine from 1 to 3.8 mg/L as Cl_2 . Results from this figure are what would be expected to be seen from chlorine disinfection and would be used by a WWTP to demonstrate that they were meeting compliance requirements.

Results of the qCPR method, seen in the second series of columns in Figure 4.4, demonstrate that the quantity of *uidA* gene or the segment of *E. coli* DNA that was

amplified was conserved throughout experimentation. This result was expected and was used as quality control to demonstrate the effectiveness of the PMA treatment. DNA is a stable molecule, and the qPCR method alone does not distinguish between DNA from viable and non-viable cells, nor does it distinguish between intracellular and extracellular DNA. For each following disinfection treatment, results of the qPCR treatment demonstrate that a consistent quantity of *E. coli* DNA was extracted for each concentration tested.

Results of the PMA-qPCR method are shown in the third series of columns of Figure 4.4. This method is designed to exclude both extracellular DNA as well as DNA from cells with damaged membranes. It quantifies the population of viable *E. coli*. Initial concentrations of viable *E. coli* were measured to be 7.12 Log CCE/mL, which were similar to results of the culture method that measured 7.59 Log CFU/mL. With chlorination, the population of viable *E. coli* was at most reduced to 5.70 Log CCE/mL, achieved with 1.9 mg/L Cl₂. When normalized to the starting quantity of *E. coli*, measured in wastewater the greatest log reduction of viable *E. coli* observed with NaOCl treatment was 1.42 (see Table 4.2).

The live/dead viability stain has been previously used to examine VBNC bacteria in a sample as well as to complement the PMA-qPCR method. The stain was performed in parallel to the rest of the treatments with the purpose of visualizing the viable cells remaining after a given disinfection treatment. The stain was applied directly to a sample. Cells are not necessarily *E. coli* but represent the heterogeneous bacterial

community in the wastewater sample. The working principle of the viability assay is similar to that of PMA-qPCR in that cells with intact cellular membranes fluoresce green which are then counted as viable or live. Cells with compromised membranes fluoresce red and counted as non-viable or dead. Results of the total live and dead cells counts are shown in Figure 4.5-a, and images of a representative field of view are shown Figure 4.5-b. Results show a similar trend as was observed with the PMA-qPCR method. There is a small decrease in the total number of cells at concentrations of 1.9 mg/L as Cl_2 and above. It is also interesting that the percent of live cells remains high throughout ranging between 100 and 88%.

Overall, results indicate that chlorine is effective at reducing culturable *E. coli* in a consistent and predictable manner which, allow WWTP to meet their compliance requirements. However, the culturability of cells is only part of the picture. Results of the PMC-qPCR technique demonstrate that a large portion of cells escape detection and survive in the VBNC-state.

Figure 4.4: Comparison results from culture methods (1st series of columns), qPCR (2nd series of columns), and PMA-qPCR (3rd series of columns) of secondary wastewater spiked with *E. coli* and treated with NaOCl at concentrations between 1-3.8 mg/L as Cl₂ and a 30 minutes contact time

Table 4.2: Log reduction of cultured and viable *E. coli* following treatment with NaOCl at concentrations between 1-3.8 mg/L as Cl₂ and a 30 minutes contact time

Treatment NaOCl [mg/L as Cl ₂]	Log Reduction of <i>E. coli</i>	
	Cultured	PMA-qPCR (viable cells)
1.0	3.35	0.20
1.9	5.82	1.42
2.8	6.05	1.19
3.8	7.14	0.81

Figure 4.5: (a) Percent of live and dead cells as well as total number of cells as determined by the live/dead viability stain of secondary wastewater spiked with *E. coli* after treatment with NaOCl concentrations of 1-3.8 mg/L as Cl₂ and a 30 minute contact time (b) corresponding images of the live/dead viability stain from a representative field of view

4.3.2 Ultraviolet light

UV fluences from 10 and 60 mJ/cm² were studied and results presented in Figure 4.6. Results of the culture method, shown in the first series of columns, show a decrease in the concentration of culturable *E. coli* from an initial concentration of 7.64 Log CFU/mL to 1.10 Log CFU/mL. The curve demonstrates what is expected to be seen with culture methods following UV disinfection; a decrease in culturable cells with increasing fluences with the tailings effect occurring with fluences of 40 mJ/cm² and above. Results of the qPCR and the PMA-qPCR are shown in the second and third series of columns of Figure 4.6, respectively. Results of the latter two methods show a similar trend. The concentration of total *E. coli*, as measured by qPCR, decreased from 7.62 to at most 6.66 Log CCE/mL, representing an 80% reduction of cells. A similar reduction of viable *E. coli*, as measured with the PMA-qPCR has been observed. Viable *E. coli* were reduced by 89% with concentrations fell from 7.53 to 6.56 Log CCE/mL. This similarity in trend may at first suggest that UV was not effective at reducing VBNC bacteria. However, UV inactivates microorganisms by creating pyrimidine dimers in the nucleic acid of cells, which changes the DNA sequence without affecting membrane permeability rendering the PMA treatment void. Similar observations have been made by other researchers who have used these changes to the DNA sequence to measure the extent of UV-induced damage to cells. For example, researchers used differences in results of the qPCR to examine changes in long fragments of DNA (10³-10⁴ bp) that occurred in human genes exposed to 100 mJ/cm² UV (Van Houten et al., 2000). Another study applied results of the qPCR similarly to study UV-induced lesions of adenovirus DNA following

low and medium pressure UV (Eischeid et al., 2009). Different lengths of DNA fragments have also been used to monitor the repair of UV-induced DNA-damage of bacterial communities of wastewater such as *Pseudomonas aeruginosa* and *Enterococcus faecium* (Süß et al., 2009).

Results of the live/dead viability stain, shown in Figure 4.7-a and Figure 4.7-b, demonstrate a similar trend to results of qPCR and PMA-qPCR. There was both little changes to the total number of cells, ranging from 70 to 90 cells, after each UV fluence as well as to the percent of live cells (shown in green), ranging from 90- 98%. The working principle of the PMA-qPCR and the live/dead viability stain are similar insofar, that damage to the cell membrane is required for a cell to be either counted as non-viable as is the case with the PMA-qPCR method or as dead (red) as is the case with the live/dead viability stain. The deactivation of cells with UV light is a physical process where the membrane integrity of cells remains unaffected. When measured with the PMA-qPCR as well as the live/dead viability stain, cells treated with UV would be expected to maintain membrane integrity and remain unchanged.

Figure 4.6: Comparison of results from the cultured methods (1st series of columns), qPCR (2nd series of columns), and PMA-qPCR (3rd series of columns) of secondary wastewater spiked with *E. coli* and treated UV fluences between 10-60 mJ/cm²

Figure 4.7: (a) Percent of live and dead cells as well as total number of cells as determined by the live/dead viability stain of secondary wastewater spiked with *E. coli* after treatment with UV fluences between 10-60 mJ/cm^2 (b) corresponding images of the live/dead viability stain from a representative field of view

4.3.3 Hydrogen peroxide

Figure 4.8 presents and compares results of the three testing methods utilized in this study after treatment with hydrogen peroxide. The first series of columns show the results measured by culturing where a decrease in culturable cells with increasing concentrations of HP. Initial culturable cell concentrations of 7.59 Log CFU/mL were reduced to 2.20 Log CFU/mL with 10 g/L HP and a contact time of 30 minutes. The second series of columns demonstrate results of the qPCR without PMA treatment and confirms that DNA was conserved. The third set of columns show results of the combined PMA-qPCR method. With this approach, the concentration of viable *E. coli* was decreased from 7.49 Log CCE/mL to at most 6.21 Log CCE/mL achieved with the highest concentration of 10 g/L HP with a corresponding log-reduction of viable *E. coli* of 1.28 (see Table 4.3). Results of the live/dead viability staining indicate some variation between the quantities of live cells counted between the samples. However, the proportion of dead cells remains similar throughout testing (see Figure 4.9).

As a disinfectant, HP (H_2O_2) has many desirable qualities. It is stable in liquid form and has an oxidation-reduction potential (ORP) 1.78 volts, or 1.3 times that of chlorine at 1.49 volts. However, the use of HP is kinetically limited, which is reflected in the low performance of HP as a disinfectant. For example, in a paper that studied the use of HP for disinfection found that 100-250 mg/L of HP with contact times of 2 hours would be required to reduce fecal coliform concentrations by 2 Log CFU/mL (Wagner et al., 2002). This represents a 20-fold increase in the concentration of HP compared to that of chlorine to achieve the same level (Tchobanoglous et al., 2003). Results of the research

presented here show that although the culturability of *E. coli* was reduced, a large portion cells remain viable, further contributing to the case that hydrogen peroxide is not a desirable alternative to chlorine.

Figure 4.8: Comparison of results from the cultured methods (1st series of columns), qPCR (2nd series of columns), and PMA-qPCR (3rd series of columns) of secondary wastewater spiked with *E. coli* and treated with HP at concentrations between 1-10 g/L and a 30 minutes contact time

Table 4.3: Log reduction of cultured and viable *E. coli* following treatment with HP at concentrations between 1-10 g/L and a 30 minutes contact time

Treatment HP [g/L]	Log reduction of <i>E. coli</i>	
	Cultured	PMA-qPCR (viable cells)
1	0.71	0.40
2.5	2.55	0.29
5	4.53	0.59
10	5.39	1.28

(b)

Figure 4.9: (a) Percent of live and dead cells as well as total number of cells as determined by the live/dead viability stain of secondary wastewater spiked with *E. coli* after treatment with HP concentrations of 1-10 g/L and a 30 minute contact time (b) corresponding images of the live/dead viability stain from a representative field of view

4.3.4 Peracetic acid

Wastewater was disinfected with PAA at concentrations ranging from 0.58-4.46 mg/L PAA. Samples were analyzed with culture methods, qPCR, PMA-qPCR, as well as with live/dead viability stain. The first series of columns, presented in Figure 4.10, demonstrate results of the culture method and shows a similar decrease in culturable cells to those observed with NaClO. PAA reduced culturable *E. coli* from 7.60 Log CFU/mL to 1.24 Log CFU/mL with 4.64 mg/L PAA and a corresponding log-reduction of culturable *E. coli* of 6.36 (Table 4.4). The second series of columns show results of the qPCR and demonstrate that DNA measured from samples was conserved. The third set of columns present results from the qPCR-PMA method and shows an increasing reduction in viable *E. coli* from 7.69 Log CCE/mL to at most 6.19 Log CCE/mL with 4.65 mg/L PAA or a log reduction of viable *E. coli* of 1.5 (Table 4.4). Results from the live/dead viability stain, Figure 4.11-a, and Figure 4.11-b. Figure 4.11-a demonstrate that the total number of cells stayed consistent ranged between 75 and 95 cells. However, the percent of dead cells significantly increased at 4.64 mg/L PAA, the highest concentration tested, indicating that higher concentrations of the chemical treatment might be more effective at reducing VBNC bacteria.

As a wastewater disinfectant, PAA is considered a viable alternative to chlorine disinfection (US EPA, 2017; Wagner et al., 2002). It offers many advantages including short contact times, effectiveness over wide a pH and temperature range, and has been shown to be a good bactericide and a virucide (Lazarova et al., 1998; Rajala-Mustonen et al., 1997; Wagner et al., 2002). It has an ORP of 1.76 volts, which slightly higher than

that of chlorine at 1.49 volts. For its working mechanisms, it is thought that PAA affects the functioning of the cytoplasmic membrane, which leads to rupturing if enough damage is caused (Kitis, 2004; Rutala and Weber, 2008; US EPA, 2012). Comparing results of NaOCl with those of PAA, both disinfectants showed a similar log-reductions of culturable *E. coli* were 3.8 mg/L as Cl achieved a 7.14 log reduction and 4.6 mg/L of PAA achieved 6.36 log reductions. An additional 0.8 log reduction was obtained with PAA. The log reductions of viable *E. coli* were also comparable. The greatest log-reduction observed with NaOCl was 1.42, achieved with 1.7 mg/L as Cl, in comparison to 1.50 log reduction achieved with 4.6 mg/L PAA; a difference of only 0.08 logs. Results of the live/dead viability staining also showed a similar trend with both disinfectants. Little change was observed to the total number cells as well as the percent live cells following treatment except the highest concentration of PAA of 4.64 mg/L. At this concentration, the percent live cells decreased to approximately 16%. This latter result suggests that higher concentrations of PAA may have a greater effect on the integrity of the cell membrane. It is also possible that higher concentrations of oxidants than those tested would show more significant gains in the reduction of viable *E. coli*.

Figure 4.10: Comparison of results from the culture methods (1st series of columns), qPCR (2nd series of columns), and PMA-qPCR (3rd series of columns) of secondary wastewater spiked with *E. coli* and treated with PAA at concentrations between 1.16-4.64 mg/L and a 30 minutes contact time

Table 4.4: Log reduction of cultured and viable *E. coli* following treatment with PAA at concentrations between 1.16-4.64 mg/L and a 30 minutes contact time

Treatment	Log reduction of <i>E. coli</i>	
	Cultured	PMA-qPCR (viable cells)
0.6	0.26	0.24
1.2	5.40	0.89
2.3	4.71	1.09
4.6	6.36	1.50

Figure 4.11: (a) Percent of live and dead cells as well as total number of cells as determined by the live/dead viability stain of secondary wastewater spiked with *E. coli* after treatment with PAA concentrations of 1.16-4.64 mg/L and a 30 minute contact time (b) corresponding images of the live/dead viability stain from a representative field of view

4.3.5 Ferrate(VI)

The research presented in this section investigated ferrate(VI) concentrations between 9 and 54 mg/L as Fe(VI) for the reduction of *E. coli* in wastewater using culturing, the direct use of qPCR and the combined use of PMA and qPCR, see Figure 4.12. Results of culture method, shown in the first series of columns of Figure 4.12, demonstrate a reduction in culturable cells from 7.66 Log CFU/mL to 1.45 Log CFU/mL with 9 mg/L as Fe(VI) to 0.79 Log CFU/mL with 54 mg/L as Fe(VI). Results of the qPCR presented in the second series of columns range between 7.72 and 8.59 Log CCE/mL and represent the total quantity of DNA extracted after each treatment without PMA treatment. The third set of columns quantifies viable *E. coli* as measured by the PMA-qPCR method. Results show a reduction from 8.62 Log CCE/mL to 5.29 Log CCE/mL measured at a concentration of 54 mg/L as Fe(VI). Results of the live/dead viability stain are shown in Figure 4.13, and both the cell quantification (Figure 4.13-a) and corresponding images (Figure 4.13-b) differ from previous disinfection presented in this study. A decreasing trend of the total number of cells as well as the percent of live cells is observed with increasing concentrations of ferrate(VI) (Figure 4.13-a). Corresponding images show groupings of cells or flocs that appeared due to ferric or Fe(III) residuals (Figure 4.13-b). At 9 mg/L as Fe(IV), three flocs are observed composed primarily live cells, as indicated by green fluorescing cells. As the concentration of ferrate(VI) is increased, the concentration of live bacteria within a floc decreases. This suggests that either disinfection occurs before formation of the floc or that ferrate(VI) can penetrate flocs to reach the entangled bacteria. As the concentration increased to 27 mg/L as Fe(VI), a

greater number of flocs was observed containing primarily red-fluorescing cells indicating membrane-damaged or dead cells. At the highest concentrations of 36 and 54 mg/L as Fe(VI), lower overall quantities of cells were observed as well as a lower number of flocs, suggesting that the number of bacteria entangled within flocs was reduced and fewer flocs were able to be visualized with fluorescence.

Potassium ferrate (K_2FeO_4) is a commercially-available salt that readily dissolves in water to produce the ferrate(VI) ion, FeO_4^{2-} . Ferrate(VI) has a +6 valence state and is the strongest oxidant of the study with an ORP of 2.2 volts (Sharma, 2002). However, similarly to HP, the ORP is only part of the story. The speciation of ferrate(VI) into the protonated ($HFeO_4^-$) and un-protonated (FeO_4^{2-}) form has a pKa between 7.2 and 7.3 (Sharma, 2008; Tiwari et al., 2007), where the protonated form is a stronger oxidant. Once oxidized, ferrate(VI) is reduced to ferric iron which has a +3 valence state and would act as would wastewater flocculent ferric chloride (Bandala et al., 2009; Sharma, 2002). Consequently, researchers have investigated the use of ferrate(VI) as a combined disinfectant and flocculent and were able to achieve between 3 and 6 Log reduction of bacteria with 28-48% removal of organic matter (Bandala et al., 2009; Gombos et al., 2012; Jiang et al., 2009). The formation of flocs and the clustering of bacteria was observed with the live/dead viability stain. Research that investigated the use of chlorine dioxide has shown that bacteria entrapped in particles may survive disinfection (Narkis et al., 1995). However, it has also been found that NaOCl was able to penetrate the macro and micro pores of wastewater particles to disinfect coliform bacteria that were entrapped within the particles (Dietrich et al., 2003). In the case presented here,

flocs are formed during disinfection, but the formation of flocs does not appear to hinder the disinfection process. The disinfection mechanism of ferrate(VI) has been less studied compared to the other disinfectants within the study. However, one study investigated the working mechanism of several disinfectants by observing protein release, lipid peroxidation, changes to cell permeability, damage to the intracellular enzymes, and morphological changes of cells (Cho et al., 2010). The study observed that the strength of the oxidant affects the disinfection mechanism (Cho et al., 2010). Stronger oxidants such as ozone had greater effects on the cell wall permeability while weaker oxidants such as free chlorine had more of an effect on intracellular components (Cho et al., 2010). Ferrate(VI) has an ORP similar to that of ozone, which has an ORP of 2.02 volts. By extension, it could be theorized that ferrate(VI) has a greater effect on the cell wall permeability than weaker oxidants. It is also important to note that the concentrations of ferrate(VI) used in the current study were higher than those used with other oxidants such as NaOCl and PAA. The purpose of the study was to examine the populations of culturable and viable *E. coli* following selected disinfection treatments. As such, a literature survey was performed to determine the concentration ranges of disinfectants that were reported. Results of the culture experiments were also used as a form of quality control to assure that a similar level of disinfection was achieved with each treatment. Culture experiments with ferrate(VI) show a similar trend to other disinfectants studied; decreasing populations of culturable *E. coli* are observed with increasing chemical concentrations. At 27 mg/L Fe(VI) and above ferrate(VI) ferrate

showed the greatest log reductions of viable *E. coli* of the study, which may be due to the strength and mechanism of ferrate(VI).

Figure 4.12: Comparison of results from the culture methods (1st series of columns), qPCR (2nd series of columns), and PMA-qPCR (3rd series of columns) of secondary wastewater spiked with *E. coli* and treated with ferrate(VI) at concentrations of 9-54 mg/L as Fe(VI) and a 30 minutes contact time

Table 4.5: Log reduction of cultured and viable *E. coli* following treatment with ferrate(VI) at concentrations of 9-54 mg/L as Fe(VI) and a 30 minutes contact time

Treatment	Log reduction of <i>E. coli</i>	
Ferrate(VI) [mg/L]	Cultured	PMA-qPCR (viable cells)
9	6.21	0.49
27	6.35	1.73
36	6.52	2.65
54	6.86	3.33

Figure 4.13 a) Percent of live and dead cells as well as total number of cells as determined by the live/dead viability stain of secondary wastewater spiked with *E. coli* after treatment with ferrate(VI) concentrations of 9-54 mg/L as Fe(VI) and a 30 minute contact time (b) corresponding images of the live/dead viability stain from a representative field of view

4.4 Conclusion

This study compared culturable and viable *E. coli* in wastewater following a number of disinfection treatments including NaOCl, HP, PAA, ferrate(VI), and UV at concentrations or fluences that would be used at WWTPs or that were suggested in the literature. With the exception of UV, the use of the PMA-qPCR method demonstrated that a large portion of *E. coli* in wastewater remain undetected by culture methods and are being released in a VBNC state. Viable *E. coli* was reduced by 1.4 logs with NaOCl, by 1.5 logs with PAA, and 3 logs ferrate(VI), while the culturability of was reduced to between 6.4 and 7 logs for all treatments. NaOCl and PAA have similar ORPs and disinfection mechanisms. That is, both chemicals are more likely to permeate the cell membrane and have an effect on internal cell components. The similarity in disinfection mechanism may also account for the similarity in reduction in viable bacteria. Ferrate(VI) is a stronger oxidant and may have a greater effect on the permeability of the cell membrane and a greater affect at reducing VBNC bacteria. However, higher concentrations of ferrate(VI) were used in the study in comparison to those of NaOCl and PAA. The use of higher concentrations of oxidants should be further investigated in future studies. The PMA-qPCR method was not able to detect VBNC-bacteria following UV disinfection due to the working mechanism of UV that leaves the cell membrane intact.

4.5 References

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Chapter 5

5 Sequential disinfection of municipal wastewater with ultraviolet irradiation (UV) and ferrate(VI), and impact on wastewater quality

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Abstract

This study investigated the sequential use of UV and ferrate(VI) for the disinfection of secondary wastewater as an alternative to the application of UV or ferrate(VI) alone. Batch disinfection experiments determined that UV fluence of 40 mJ/cm² and above, provided 99.99% reduction in coliform bacteria. Concentrations of 9 mg/L of ferrate(VI) and above, provided 99.9% reduction in coliform bacteria. Placing ferrate(VI) before UV provided an additional 80% reduction in coliform bacteria compared to what was achieved with UV alone. Ferrate(VI) residuals have coagulant properties, and increases in turbidity by 95.6% were observed as was a decrease in the total number of particles and an increase in particle size due to the flocking of colloidal particles. Sequentially treated wastewater also exhibited an additional increase in COD of up to 32% when compared to the use of UV alone. No significant changes to other wastewater characteristics, such as pH, phosphorus, sulfate, and inorganic nitrogen, were observed. The sequential disinfection of wastewater with UV and ferrate(VI) has the potential to

allow for increased reductions in bacteria while maintaining effluent standards providing WWTP with additional operational flexibility.

Keywords: Sequential disinfection, ferrate(VI), potassium ferrate(VI), UV, ultraviolet light, wastewater disinfection

5.1 Introduction

UV disinfection of wastewater is a physical disinfection method that leaves no chemical residual, thereby eliminating the possible formation of chlorinated disinfection by-products (DBPs) typically associated with the use of chlorine-based disinfectants. Additionally, UV has been shown to be effective against chlorine-resistant protozoa such as *Cryptosporidium parvum* oocysts and *Giardia lamblia* (Bukhari et al., 1999; Dell’Erba et al., 2007; Liberti et al., 2003; Linden et al., 2001). UV disinfection relies on the transmission of light reaching microorganisms and can be hindered by suspended solids and particles in wastewater. With increasing frequency and intensity of severe weather events, wastewater treatment plants (WWTP) are in need of disinfection strategies that can offer increased operational flexibility and efficiencies.

Recent research has shown that the combined or sequential use of two disinfectants may have synergistic effects. For example, the sequential use of chlorine dioxide and free chlorine has been demonstrated to increase the inactivation of spore-forming bacteria *Bacillus subtilis* (Cho et al., 2006a). Synergistic effects have also been observed when combining chlorine and ozone (Rennecker et al., 2000) as well as chlorine and UV

(Murphy et al., 2008). To reduce the risk of forming halogenated DBPs, research has also investigated combining UV with hydrogen peroxide (Cho et al., 2011; Koivunen and Heinonen-Tanski, 2005; Vankerckhoven et al., 2011), UV and iodine (Pennell et al., 2008), UV and peracetic acid (Koivunen and Heinonen-Tanski, 2005), and UV and ozone (Jung et al., 2008).

Ferrate(VI) is a strong oxidant with an oxidation-reduction potential of 2.20 volts at pH values below 8 and 0.72 volts at pH values above 8 see Equation 5.1. It has similar potential to that of ozone that has an ORP of 2.07 volts (Jiang et al., 2001). Ferrate(VI) contains iron at a +6 valence state and spent ferrate(VI) leaves residuals of ferric oxides and hydroxides that containing iron in a +3 oxidation state, which act as a coagulant, see Equation 5.2 (Farooq and Bari, 1986; Jiang et al., 2009, 2006; Murmann and Robinson, 1974). Researchers have investigated the use of ferrate(VI) for different stages of wastewater treatment as well as the use of ferrate(VI) as a combined coagulant and disinfectant (Bandala et al., 2009; Gombos et al., 2012; Jiang et al., 2009, 2006; White and Franklin, 1998). It has also been suggested for wastewater reuse for wetland and groundwater restoration (Reimers et al., 2007) and as a disinfectant for ballast water (Jessen et al., 2006). As a disinfectant aid to UV, ferrate(VI) is an ideal candidate because it does not create the potential for halogenated DBPs typically associated with chlorine (Schuck et al., 2006). In fact, ferrate(VI) has been shown to aid in the oxidization of contaminants such as antibiotics (Sharma et al., 2006), endocrine disrupting compounds (Li et al., 2008; Srisawat et al., 2010; Yang et al., 2012) and other pharmaceuticals (Jiang

et al., 2013), as well as cyanide-containing compounds (Sharma et al., 2008) and other organic compounds (Avisar et al., 2010; Lee and von Gunten, 2010).

The purpose of this study was to investigate the sequential use of UV and ferrate(VI) for the disinfection of secondary wastewater as an alternative to the application of UV or ferrate(VI) alone. The paper evaluated the synergistic effects of the sequential use of UV and ferrate(VI) using coliform bacteria and compared the disinfection efficiencies to those obtained with UV and ferrate(VI) treatment alone. In addition, the impact of the sequential use of UV and ferrate(VI) disinfection on wastewater quality was investigated by monitoring the changes in the physical and chemical parameters of wastewater.

5.2 Materials and methods

5.2.1 Effluent wastewater samples

Effluent secondary wastewater was collected from the secondary clarifiers at a treatment plant in Ontario, Canada. The treatment plant has a 400 ML/d capacity and follows a conventional treatment which includes coarse screening, fine screening, grit removal, primary sedimentation, activated sludge, secondary sedimentation as well as year-round chlorination with sodium hypochlorite and dechlorination with sodium

thiosulfate. Samples were collected weekly in sterile 4-L high-density polyethylene containers and stored at 4°C until used.

5.2.2 Ferrate(VI)

Ferrate(VI) stock solutions were created by dissolving of 1 g/L of potassium ferrate(VI) salts, K_2FeO_4 , (Sigma-Aldrich, MO, USA, >90%) in ultrapure water (Millipore Direct-q UV 3). Stock solutions had a pH of 11 and were stored in pre-sterilized dark glass bottles at 4°C until use. The concentration of the ferrate(VI) ion, FeO_4^{2-} (noted as Fe(VI)), was verified using the direct colorimetric method (Luo et al., 2011). A 10% loss of the ferrate(VI) ion in the stock solution was observed after three hours, and stock solutions were discarded after this time. All ferrate(VI) experiments were performed in well-mixed batch reactors without pH adjustments and maintained a contact time of 30 minutes. Quenching was performed with a solution of 0.02% (w/v) sodium thiosulfate. Samples were taken from the well-mixed reactor with no additional settling time provided.

5.2.3 Ultraviolet light

A bench-scale collimated beam apparatus was used for all UV irradiation experiments. Experimental procedures and germicidal fluence calculations followed the procedure outlined by Bolton and Linden (2003) for low-pressure UV lamps and suspension depth less than 2 cm (Bolton and Linden, 2003). In brief, 150 mL wastewater was placed in tall-walled sterile glass Petri dishes and placed on a mixing plate with a magnetic stirrer that also served as the irradiation state which allowed continuous mixing within the UV reactor. A set of four low-pressure mercury lamps (Phillips UV-C germicidal lamps)

provided nearly-monochromatic UV light at 253.7 nm, and two apertures placed below the lamps focused the beam. The inside of the apparatus was painted with a matt black paint to assure little to no reflectivity from surfaces. The absorption coefficient at 254 nm was measured with a UV-Visible spectrophotometer (Varian Model Cary 100BIO) using a 1-cm quartz cuvette and radiometer (International Light NIS Traceable Radiometer/Photometer Model IL 1399A). Irradiance was measured before every experiment.

5.2.4 Culture methods

Indigenous coliform bacteria in the wastewater were used as the test organism for the duration of the study using the membrane filtration Protocol 9222 B (Eaton et al., 2005). M-Endo agar–Difco™ LES by BD Biosciences (Mississauga, ON) selective agar was used for coliform enumeration incubated 24 ± 2 hours at 37°C. Colonies with a metallic sheen were counted as positive. Membrane filtration units by Advantec (Japan) along with 0.45 μm , 47 mm S-Pak™ membrane filters (Billerica, MA, USA) were used throughout (Eaton et al., 2005). Filtration was vacuum assisted with a vacuum pressure pump by Barnant Co, model number 400-1901 (Barrington, IL, USA). Each dilution was performed with a minimum of three replicates diluted in phosphate buffered solution (PBS) (Eaton et al., 2005).

5.2.5 Particle concentration and distribution

Particle analysis was performed with the DPA4100 Particle Analysis System (Brightwell Technologies, Ottawa, ON) which uses Micro-Flow Imaging™ technology to count and take pictures of particles. Samples sizes of 10 mL were carefully injected into the

sampling syringe. The system was zeroed with ultrapure water (Millipore Direct-q UV 3) (Millipore, Etobicoke, ON). Replicates were performed by passing 2 mL of the sample while continuously mixing.

5.2.6 Wastewater quality parameters

Absorbance scans and single wavelength absorbance measurements were conducted with a UV-visible spectrophotometer (Varian Model Cary 100) that was zeroed with ultrapure water with 18.2 M Ω .cm at 25°C (Millipore Direct-q UV 3) (Millipore, Etobicoke, ON). Hach colorimetric tests were used to determine the concentration of wastewater characteristics with a DR 2800 Portable Spectrophotometer. Chemical oxygen demand (COD) was determined using COD Digestion Vials High Range 20-1500 mg/L and Hach method 8000. Ammonia was determined using the AmVer™ Salicylate Test 'N Tube™ Method with Hach method 10031, and nitrite with the Nitrite TNTplus TNT839. Nitrate was measured with TNT NitraVer® X following Hach method 10020, total phosphorus with the PhosVer®3 Acid Persulfate Digestion Method 8190, and sulfate with the SulfaVer4 with Hach Method 8051. Turbidity measurements were performed with the Hach 2100AN turbidimeter. Calibration of the turbidimeter was conducted using the StableCal® Calibration.

5.2.7 Statistical analysis

One-way analysis of variance (ANOVA) was carried out to determine if a significant difference exists between the means of two or more samples. The alternative hypothesis of means that were different was accepted if the *p*-value was less than the significance level (α) of 0.05. If accepted, the Tukey test was used posthoc to separate

the means further, using the same significance level (Currell and Dowman, 2005). Statistical analysis was performed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

5.3 Results and discussions

5.3.1 Performance of individual disinfectants

Figure 5.1 demonstrates the reduction of the coliform bacteria in wastewater following treatments of UV (Figure 5.1-a) and ferrate(VI) (Figure 5.1-b). In Figure 5.1a, UV fluences of 10 mJ/cm^2 , 20 mJ/cm^2 , 40 mJ/cm^2 , 60 mJ/cm^2 achieved 2.65, 2.15, 1.76 and 1.52 log CFU/100 mL, respectively, from an initial concentration 5.09 log CFU/100 mL. A 99.99% reduction of coliform bacteria was observed with fluences of 40 and 60 mJ/cm^2 . The inactivation curve demonstrates the tailing effect that is typically seen with UV disinfection where lower UV fluences are associated with large gains in the reduction of coliform bacteria and minor gains are observed as the fluence is increased. This effect is typically associated with particles or bio-aggregates that either shield or scatter light (Emerick et al., 2000; Kollu and Örmeci, 2012; Madge and Jensen, 2006; Qualls et al., 1983).

Figure 5.1b shows the reduction of coliform bacteria in wastewater following treatment with ferrate(VI). The initial total coliform concentration of 5.21 log CFU/100 mL was reduced to 4.95, 3.45, 2.31 and 2.37 log CFU/100 mL, with ferrate(VI) concentrations of 3, 6, 9 and 18 mg/L Fe(VI), respectively. A concentration of 9 mg/L Fe(VI) accounted for 99.9% reduction of coliform bacteria, and no additional gains were seen by doubling the concentration to 18 mg/L. Gombos et al. (2012) achieved a similar heterotrophic

bacteria inactivation of 99.8% in secondary effluent wastewater with 5 mg/L Fe(VI), while also allowing for settling. Other research achieved a 6-log reduction of total and fecal coliforms with 5 mg/L Fe(VI) with a shorter contact time of 5 minutes (Bandala et al., 2009). However, in their study, ferrate(VI) treatment was followed by a filtration step which could account for the larger removals observed (Bandala et al., 2009). Jiang et al. (2006) conducted experiments with raw influent wastewater that required a larger ferrate(VI) concentration of 15 mg/L Fe(VI) followed by sedimentation to achieve 4-log removal of fecal and total coliforms.

Figure 5.1: Inactivation of coliform bacteria in secondary wastewater subsequent to (a) UV fluences of 0, 10, 20, 40 and 60 mJ/cm² and (b) ferrate(VI) at concentrations of 0, 3, 6, 9 and 18 mg/L as Fe(VI)

5.3.2 Effect of ferrate(VI) on absorbance

Figure 5.2 presents the UV-visible absorption spectra of secondary wastewater before and after treatment with 3-18 mg/L Fe(VI). Ferrate(VI) stock solutions are purple in colour and have an absorbance peak at 510 nm. Spent ferrate is reduced to compounds

with lower oxidation states such as ferrate(V), ferric hydroxides(III), and ferric iron(III), which have an effect on the absorbance spectra in the ultraviolet (<400nm), violet (400-420 nm) and indigo (420-440 nm) ranges (Bielski and Thomas, 1987). Following the Beer-Lambert Law, increasing the ferrate(VI) concentration increased the absorbance of the samples (see Figure 5.2). Of particular interest was the increase in absorbance at 254 nm. When considering sequential disinfection treatment that involves low-pressure UV, a change in absorbance at 254 nm will affect UV exposure time required to achieve a given UV fluence. Figure 5.3 demonstrates that increasing the concentration of ferrate(VI) increases the absorbance at 254 nm and proportionally increase UV exposure times. Considering a UV fluence of 40 mJ/cm^2 , before the addition of ferrate(VI), exposure time would be 6 minutes and 34 seconds for a wastewater sample. Adding 9 mg/L ferrate(VI) would double the absorbance at 254 nm and double the exposure time to 13 minutes. UV fluences reported in subsequent experiments used the initial absorbance of the wastewater samples for the calculation of the UV dose.

Figure 5.2: UV-Visible absorbance spectra of secondary effluent wastewater treated with 0 to 18 mg/L as Fe(VI)

Figure 5.3: UV absorbance at $\lambda = 254$ nm of secondary effluent wastewater treated with 0 to 18 mg/L Fe(VI) (primary axis) and corresponding exposure time for UV fluence of 40 mJ/cm^2 (secondary axis)

5.3.3 Sequential disinfection with UV and ferrate(VI)

Figure 5.4 compares the effectiveness of the single use of UV. The use of UV being followed by 3 mg/L Fe(VI) and the use of UV followed by 9 mg/L Fe(VI) as measured by the inactivation of total coliform bacteria. The concentration of 9 mg/L ferrate(VI) was selected based on the results presented in Figure 5.1-b, where the concentration of 9 mg/L Fe(VI) had the greatest effect on reducing coliform bacteria. Further increases in ferrate(VI) had no further improvement in the reduction of coliform bacteria. Results presented in Figure 5.4 show that sequential treatment of UV followed by 3 mg/L Fe(VI) had similar results to that of using UV alone. Increasing the concentration of ferrate(VI) to 9 mg/L increased the reduction of coliform bacteria by 43-57% compared to using UV alone. The greatest gains of 57% were achieved using a UV fluence of 40 mJ/cm² where the concentration of coliform bacteria was reduced from 1.4 log CFU/100 mL (UV alone) to 0.6 log CFU/mL (UV + 9 mg/L Fe(VI)).

Figure 5.5 compares the effect of placing ferrate(VI) before or after UV in a sequential disinfection regime where UV fluences of 0, 10, 20 and 40 mJ/cm² are coupled with 9mg/L Fe(VI). As mentioned, the sample absorbance required to calculate the UV fluence was measured before the addition of ferrate(VI). Figure 5.5 demonstrates that the order of the UV and ferrate(VI) application has no significant effect on the inactivation of coliform bacteria. At a UV fluence of 10 mJ/cm², applying ferrate(VI) before UV resulted in a slightly lower inactivation of coliform likely due to the increase in sample absorbance, but the difference quickly disappeared at higher UV fluences of 20 and 40 mJ/cm².

Figure 5.4: Inactivation of total coliform bacteria following UV disinfection using fluences of 10, 20, 40 and 60 mJ/cm² and sequential disinfection of secondary wastewater using the same UV fluences followed by ferrate(VI) concentrations of 3 or 9 mg/L as Fe(VI)

Figure 5.5: Inactivation of total coliform bacteria following sequential treatment of UV fluences of 10, 20, and 40 mJ/cm² with 9mg/L ferrate(VI) or sequential treatment of 9mg/L ferrate(VI) and UV fluences of 10, 20, and 40 mJ/cm²

5.3.4 Particle concentration and size distribution

Results of turbidity and particle concentration measurements are shown in Figure 5.6. The turbidity of secondary effluent wastewater was first measured to be 6.00 NTU and remained unchanged after UV, see Figure 5.6-a. The use of ferrate(IV) increased the turbidity to 11.73 NTU, representing a 95% increase. The sequential treatments of UV and ferrate(VI) further increased turbidity to 13.13 NTU when UV was placed first and later to 13.07 NTU when ferrate(VI) was placed first representing an 119% and 118% increase, respectively. Ferrate residuals include ferric and ferric hydroxide containing iron in a +3 oxidation state, see Equations 5.2 and 5.3, which acts as a coagulant (Jiang et al., 2009). The increase in turbidity can be attributed to the formation of aggregates due to ferrate residuals.

Figure 5.6-b shows the total number of particles per volume of sample after each treatment. Results show an initial particle concentration of approximately 68,000 particles/mL. UV treatment slightly increased the number of particles to approximately 76,000 particles/mL. The increase in the concentration of particles may be due to mixing during UV exposure and breaking up naturally formed flocs in wastewater. The addition of ferrate(VI) to wastewater in any combination decreased the particle concentration to between 26,000 to 54,000 particles/mL. The coagulation effect of ferrate residuals and the agglomeration of smaller particles into larger flocs reduced the number of particles in a sample. This effect is further seen in Figure 5.7 which shows the change of the particle size distribution after each treatment.

Figure 5.7 shows the particle size distribution after each treatment. For every treatment, between 81-99% of all particles counted had equivalent circular diameters of 20 μm or less. Samples of initial wastewater and following UV showed similar trends with 99% of particles being smaller than 20 μm . In addition, the number of particles counted decreased as the particle size increased. This trend can also be seen in the flow cell images shown in Figure 5.8a-b. The addition of ferrate(VI) was accompanied by an increase in particles with larger diameters. The contact time of ferrate(VI) also appeared to influence the particle size distribution. The longer ferrate(VI) was allowed to mix in wastewater, the greater the concentration of larger particles was. This can be observed by comparing the particle size distribution of the use of ferrate(VI) alone to that of UV followed by ferrate(VI) and that of ferrate(VI) followed by UV. The contact time for the latter treatment regime was the contact time used for ferrate(VI) added to that of the contact time for UV fluence of 40 mJ/cm^2 . Even though ferrate(VI) was quenched after a set amount of time, residual ferric ions and ferric hydroxides remain in solution during UV irradiation. This allowed ferric compounds additional time to aggregate colloidal material and increased particles sizes. This effect can also be seen in Figure 5.8 c-e.

Figure 5.6: Average (a) turbidity and (b) total particle concentration - of secondary effluent wastewater treated with UV fluence of 40 mJ/cm^2 , 9 mg/L Fe(VI) , sequential treatment of UV fluence of 40 mJ/cm^2 followed by 9 mg/L Fe(VI) and sequential treatment of 9 mg/L Fe(VI) followed by UV fluence of 40 mJ/cm^2

Figure 5.7: Particle size distribution of secondary effluent wastewater treated with UV fluence of 40 mJ/cm², 9 mg/L Fe(VI), sequential treatment with UV fluence of 40 mJ/cm² and 9 mg/L Fe(VI) and sequential treatment of 9 mg/L Fe(VI) and UV fluence of 40 mJ/cm²

Figure 5.8: Flow cell images of (a) secondary effluent wastewater treated with (b) UV fluence of 40 mJ/cm^2 , (c) 9 mg/L Fe(VI) , (d) sequential treatment with UV fluence of 40 mJ/cm^2 and (e) 9 mg/L Fe(VI) and (f) sequential treatment of 9 mg/L Fe(VI) and UV fluence of 40 mJ/cm^2

5.3.5 Wastewater characteristics

During experiments with ferrate(VI) and UV, wastewater characteristics including pH, total chemical oxygen demand (tCOD), total phosphorus, sulfate, ammonia, nitrite, and nitrate were monitored to determine the changes and potential improvements in wastewater quality due to the oxidative characteristics of ferrate(VI). Results are shown in Figure 5.9 to Figure 5.10.

Figure 5.9-a shows the change in pH that occurred after each treatment. Overall, no significant changes in pH were observed. Initial pH was 7.46 and increased to at most 8.22 following sequential treatment of UV followed by ferrate(VI), which is in the acceptable range (Jiang et al., 2001).

The change in COD is reported in Figure 5.9-b. Initial COD was measured to be 125 ± 8 mg/L. UV treatment as well as sequential treatment of UV followed by ferrate(VI) had no significant effect on the COD of wastewater with COD concentration of 117 ± 6 mg/L ($p = 0.11$) and 133 ± 5 mg/L ($p = 0.14$), respectively. Ferrate(VI) treatment, as well as ferrate(VI), followed by UV significantly increased COD concentrations. When ferrate(VI) was applied alone, the COD was 165 ± 7 mg/L ($p = 0.001$), representing a 32% increase. A slightly lower increase of 17% was observed when UV followed ferrate(VI) with a COD of 146 ± 4 mg/L ($p = 0.032$). However, even with the increases wastewater is still of low strength. Ferrate(VI) is a strong oxidant and can oxidize organic matter resulting in a decrease in COD. However, ferrate(VI) can also labialize organic matter originally refractory to the dichromate COD and increase the COD of the sample.

Bandala et al. (2009) reported a nearly 50% reduction in total organic carbon (TOC) in wastewater with 14 mg/L Fe(VI) compared to only approximately 25% when a similar concentration of hypochlorite was used. The initial TOC of their wastewater was in the range of 80-110 mg/L. While direct comparisons of TOC and COD can be problematic, Dubber and Gray (2010) have shown a direct relationship between TOC and COD when speaking of effluent wastewater and similar trends which can be compared. Jiang et al. (2009) also observed a 40% reduction of COD when raw sewage was treated with ferrate(VI). Initial concentrations of COD in the raw wastewater used were nearly ten times higher than that of the secondary effluent wastewater used in the present study. It should be noted that both of these studies allowed for a settling time following treatment with ferrate(VI), which would have removed the organic aggregates and excluded them from the COD test.

Figure 5.9-c presents the change in total phosphorus after each treatment. No significant changes were observed, and levels remained low after each treatment varying between 1.43 and 1.56 mg/L as P.

Figure 5.9-d depicts the change of sulfate with each treatment. Sulfate levels in secondary effluent wastewater were measured at 59 ± 1 mg/L as SO_4^{2-} and varied between 56 and 62 mg/L as SO_4^{2-} depending on the treatment applied. However, treatments used did not significantly affect the sulfate levels in wastewater.

Figure 5.10 depicts the change in inorganic nitrogen with each treatment. Concentrations of ammonia ranged from 26.3 ± 0.6 mg/L $\text{NH}_3\text{-N}$ to 29.1 ± 0.2 mg/L $\text{NH}_3\text{-N}$.

N, see Figure 5.10-a. The concentration of nitrite (NO_2^-) in wastewater was measured to be $0.057 \pm 0.01 \text{ mg/L NO}_2^- \text{-N}$ and changed to at most $0.050 \pm 0.02 \text{ mg/L NO}_2^- \text{-N}$ achieved with sequential treatment regardless if UV was placed first or second, see Figure 5.10-b. Nitrate (NO_3^-) concentrations ranged from $0.1 \pm 0.1 \text{ mg/L NO}_3^- \text{-N}$ to $0.5 \pm 0.1 \text{ mg/L NO}_3^- \text{-N}$, see Figure 5.10-c. Concentrations of nitrate were low, and no significant change was observed. Disinfection treatments applied had no significant effect on the distribution of nitrogen in wastewater.

Overall, the results showed that no significant changes in wastewater quality were observed after treatment with single or combined use of UV and ferrate(VI).

Figure 5.9: Average characteristics of secondary effluent wastewater treated with UV fluence of 40 mJ/cm^2 , 9 mg/L of Fe(VI) , sequential treatment of UV fluence of 40 mJ/cm^2 followed by 9 mg/L Fe(VI) and sequential treatment of 9 mg/L Fe(VI) followed by UV fluence of 40 mJ/cm^2 (a) pH (b) tCOD (c) phosphorus, and (d) sulfate

Figure 5.10: Average concentration of nitrogen compounds in secondary effluent wastewater treated with UV fluence of 40 mJ/cm^2 , 9 mg/L of Fe(VI), sequential treatment of UV fluence of 40 mJ/cm^2 followed by 9 mg/L Fe(VI) and sequential treatment of 9 mg/L Fe(VI) followed by UV fluence of 40 mJ/cm^2 (a) ammonia-nitrogen (b) nitrite-nitrogen (c) nitrate-nitrogen

5.4 Conclusion

This research aimed to investigate the sequential use of UV and ferrate(VI) for the disinfection of secondary wastewater as an alternative to the application of UV or ferrate(VI) alone. The use of 40 mJ/cm^2 of UV alone was effective in reducing coliform bacteria by 99.99% while the single use of 9 mg/L as Fe(VI) achieved 99.9% reduction. Combined treatment offered an additional reduction of coliform bacteria of 80% without affecting effluent wastewater characteristics. The combined treatment of UV and ferrate(VI) increased the concentration and size of particles due to the flocculation effect of ferric iron compounds.

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Chapter 6

6 Inactivation and regrowth, reactivation and resuscitation of culturable and viable but nonculturable (VBNC) bacteria in secondary wastewater after sequential disinfection with ultraviolet light and ferrate(VI)

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Abstract

The combined use of two disinfectants may provide increased efficiencies and synergistic effects. This study investigated the use of ferrate(VI) and ultraviolet light (UV) both alone and in sequence for the disinfection of wastewater where the culturability of indicator organism *Escherichia coli* as well as spore-forming bacteria *Clostridium perfringens* were compared. In addition, the combined use of propidium monoazide (PMA) and quantitative polymerase chain reaction (qPCR) provided insights into the viable but nonculturable (VBNC) population of *E. coli*. Changes to the culturability of indigenous coliform bacteria were monitored for 48 hours to provide insight into potential regrowth, reactivation and resuscitation. Results of the study indicate that UV fluences above 20 mJ/cm² and ferrate(VI) concentrations above 9 mg/L as Fe(VI) assured compliance and reduced culturable *E. coli* to below 2 Log CFU/mL. Combining UV fluence of 40 mJ/cm² and 9mg/L of ferrate(VI) provided additional 0.9 Log reduction compared to the use of UV alone and also overcame the tailing effect typically seen with UV disinfection. Indigenous *C. perfringens* were reduced by 95.5%

with a UV fluence of 40 mJ/cm² but no reduction was observed with ferrate(VI) treatments. The combined treatment of UV fluence of 40 mJ/cm² and 9 mg/L of ferrate(VI) provided an additional 0.7 Log reduction. However, due to low concentrations of indigenous *C. perfringens* and variability in the culture method, the decrease was not found to be statistically significant when compared to the use of UV alone. A large amount of cells exiting treatments were found to enter the VBNC state. Of an initial concentration of 7.6 Log CCE/mL of *E. coli*, a total of 6.7 and 5.6 Log CCE/mL remained viable after treatment with 9 mg/L as Fe(VI) and the combined treatment of UV fluence of 40 mJ/cm² and 9 mg/L as Fe(VI) when culturable cells were below 2 CFU/mL. The study of regrowth, reactivation, and resuscitation, demonstrated that regrowth/resuscitation was observed 6 hours after treatment with sodium hypochlorite which, was the shortest time following any of the treatments studied. This result suggests that regrowth and resuscitation may play a larger role with following disinfection with sodium hypochlorite than other treatments studied. No significant increases in culturable coliform bacteria was observed following 9 mg/L as Fe(VI). Regrowth and reactivation were observed after UV fluence of 40 mJ/cm² treatment after 18 hours, which was also seen with the combined treatment of UV fluence of 40 mJ/cm² and 9 mg/L as Fe(VI). Regrowth, reactivation and resuscitation are all possible following combined treatment. However, similar to UV disinfection, reactivation is expected to have the greatest effect.

6.1 Introduction

The germicidal effects of UV have been known since the early 20th century (US EPA, 2003) and have been applied for the disinfection of air (Memarzadeh et al., 2010b), food (Koutchma, 2008), and water (Guo et al., 2009b; Hassen et al., 2000; Murphy et al., 2008; US EPA, 2003). As a wastewater disinfection technology, UV has many advantages. It eliminates the release of chemical residuals to the environment, which precludes the formation of harmful DBP-associated with chlorine, ozone or other chemical oxidants (US EPA, 2003). UV is also more effective at reducing chlorine-resistant microorganisms such as *Cryptosporidium parvum* oocysts and *Giardia lamblia* (Bukhari et al., 1999; Dell’Erba et al., 2007; Liberti et al., 2003; Linden et al., 2001).

The sequential or combined uses of certain disinfectant pairs are known to have synergistic effects. For example, the sequential use of chlorine dioxide and free chlorine has been shown to increase inactivation levels of spore-forming bacteria *Bacillus subtilis* (Cho et al., 2006a). Combining chlorine with ozone has been shown to have beneficial effects (Rennecker et al., 2000). Researchers have also investigated the use of two chlorine-free disinfectants to eliminate the risk of forming halogenated DBP-associated with the use of chlorine-containing compounds, such as combining UV with hydrogen peroxide (Cho et al., 2011; Koivunen and Heinonen-Tanski, 2005; Vankerckhoven et al., 2011), iodine (Pennell et al., 2008), and peracetic acid (Koivunen and Heinonen-Tanski, 2005). The sequential or combined use of UV with a secondary disinfectant also has the added advantage of offering more flexibility to increase disinfection performance during high flows or extreme weather events. Full-scale applications of UV disinfection can

have unique operational difficulties. For instance, the effectiveness of UV is limited by particles in wastewater which reduces the efficiency if turbidity increases. In addition, lamp performance can decrease over time and supplementing UV with a secondary disinfectant can prolong the use of lamps before replacement. Adding an oxidant upstream of UV treatment may help reduce lamp fouling by controlling bacteria and algae growth associated with fouling.

Ferrate is a strong oxidant that contains iron at a +6 oxidation state. As a wastewater disinfectant, ferrate has many desirable qualities. Disinfection residuals of ferrate include ferric iron compounds, Fe(III), which act as a coagulant to further increase the removal of suspended solids. Research has investigated the use of ferrate for different stages of wastewater treatment and has shown favorable results. For example, the use of ferrate has been shown to simultaneously act as a coagulant and disinfectant (Bandala et al., 2009; Gombos et al., 2012; Jiang et al., 2009, 2006; White and Franklin, 1998). Ferrate(VI) has also been used to oxidize emerging contaminants such as antibiotics (Sharma et al., 2006), endocrine disrupting compounds (Li et al., 2008; Srisawat et al., 2010; Yang et al., 2012), cyanide-containing compounds (Sharma et al., 2008) and other organic compounds (Avisar et al., 2010; Jiang et al., 2013; Lee and von Gunten, 2010). To date, no studies were found in the literature that have investigated the combined use of UV and ferrate.

The purpose of this study was to investigate the use of ferrate(VI) both alone and in combination with UV. The study investigated the culturability of indicator organism *E.*

coli as well as *C. perfringens*, a spore-forming bacteria that has been suggested as a surrogate for enteric viruses (Abia et al., 2015; Harwood et al., 2005; Payment and Franco, 1993; Venczel et al., 1997). In addition, there is growing evidence that disinfection treatments may induce a VBNC-state in cells where viability and virulence may be maintained (Byrd et al., 1991; Calgua et al., 2014; Noor et al., 2009; Oliver et al., 2005; Servais et al., 2009). As such, the VBNC-state of *E. coli* following treatments of UV and ferrate(VI) was investigated as was the ability of bacteria to regain culturability via regrowth, reactivation and resuscitation.

6.2 Materials and methods

6.2.1 Ultraviolet light

A bench-scale collimated beam apparatus with low-pressure UV lamps was used throughout the study. Procedures and germicidal fluence calculations followed those for suspension depth less than 2 cm (Bolton and Linden, 2003). A sample volume of 150 mL was placed in a tall-walled sterile glass petri dish and placed on a mixing plate that served as the irradiation stage, and it allowed for continuous mixing during the UV irradiation. The apparatus was equipped with four mercury LP-UV lamps (Phillips UV-C germicidal lamps) which provided nearly monochromatic UV light at 253.7 nm. Two apertures were placed below the lights to focus the beam. The inside surfaces of the apparatus were painted with a matt black paint to assure little to no reflectivity. Before every experiment, the absorption coefficient at 254 nm measured using a UV-visible spectrophotometer and 1-cm quartz cuvette. The light intensity was measured using International Light NIS Traceable Radiometer/Photometer Model IL 1399A. Intensity was

used to calculate fluence times. Average light intensity measured throughout the course of experiments was 0.370 ± 0.008 mW/cm².

6.2.2 Ferrate(VI)

Stock solutions of ferrate(VI) were created by dissolving 1 g/L of potassium ferrate salts, K₂FeO₄ (>90%, Sigma-Aldrich, St. Louis, MO, USA) in ultrapure water dispensed from the Millipore Direct-q UV 3 (Millipore Sigma, Etobicoke, ON, Canada). Stock solutions had a pH of 11 and were stored in pre-sterilized dark glass bottles at 4°C until use. The concentration of the ferrate ion, FeO₄²⁻ (noted as Fe(VI)), was verified using the direct colorimetric method (Luo et al., 2011). A 10% loss of the Fe(VI) in the stock solution was observed after three hours, and stock solutions were discarded after this time. All experiments using ferrate(VI) were performed in well-mixed batch reactors without pH adjustments with a contact time of 30 minutes. Quenching was performed with a solution of 2.0 % (w/v) sodium thiosulfate. Samples were taken directly from the batch reactors with no additional settling time provided.

6.2.3 Culture methods

Membrane filtration procedure outlined in Protocol 9222B of *Standard Methods* (Eaton et al., 2005) was used to enumerate each bacteria type listed below using membrane filtration units (Advantec, Japan) along with 0.45 µm, 47 mm S-Pak™ membrane filter (Millipore Sigma, Etobicoke, ON, Canada). Filtration was vacuum assisted with a vacuum pressure pump model number 400-1901 (Barnant Co., Barrington, IL, USA). Each dilution was performed with a minimum of three replicates diluted in PBS (Eaton et al., 2005). Details of the agars used are described in the following sections.

6.2.3.1 *Escherichia coli*

E. coli are found in the intestinal tract of warm-blooded animals and often used as indicators of fecal contamination. Experiments conducted with *E. coli* K-12 strain 3000-141, ATCC® 19853 (Cedarlane Laboratories, Burlington, ON, Canada) which was spiked into unchlorinated secondary effluent collected locally from the wastewater treatment plant. The plant follows a conventional treatment train (primary sedimentation, activated sludge, secondary sedimentation) and treats approximately 390,000 m³/year. *E. coli* suspensions used during experiments were created by reviving the frozen culture of *E. coli* (10⁹ CFU/mL) overnight at 37°C in Bacto™ Tryptic Soy Broth Soybean-Casein Digest (BD, Mississauga, ON, Canada) in a ratio of 1:100. After overnight incubation, suspensions were washed 3 times by centrifuging the suspension for 5 minutes at 8,000xg, discarding the supernatant and suspending the pellet in phosphate buffered solution (PBS) (Eaton et al., 2005). Experimental suspensions were created by adding 20 mL of the washed *E. coli* suspension to 1 L of secondary effluent wastewater to achieve initial *E. coli* concentrations of 10⁷ CFU/mL. The concentration of indigenous *E. coli* was approximately 10⁶ CFU/mL and therefore spiked *E. coli* allowed for concentrations that were 1 Log higher and allowed for a consistent comparison of results that occurred over a number of weeks. Each dilution was performed with a minimum of three replicates diluted in PBS. Bacto™ EC Medium with MUG (BD, Mississauga, ON, Canada) with 1.5% (w/v) Agar Laboratory Grade ARG003.500 (Bishop, Burlington, ON, Canada) added. Plates were incubated at 35°C for 24 ± 2 hours. Positive *E. coli* colonies were fluoresced under black light.

6.2.3.2 *Clostridium perfringens*

CP Chromo Select Agar with M-CP selective Supplement I (Fluka analytical, St. Louis, MO, USA) was used to enumerate *C. perfringens* in this study. Plates were incubated anaerobically for 24 ± 2 hours at 44°C followed by an additional 2 ± 1 hour in aerobic condition at the same temperature. Anaerobic conditions were achieved with anaerobic jars with OXOID AnaeroGen anaerobic gas packs and conditions assured with OXOID Anaerobic Indicator (Thermos Scientific, Eugene, OR, USA).

6.2.3.3 *Coliform Bacteria*

Coliform bacteria are a group of rod-shaped, Gram-negative bacteria which include *E. coli* and are often used for compliance monitoring at wastewater treatment plants (WWTP). M-Endo agar–Difco™ LES (BD, Mississauga, ON, Canada) was used to culture coliform bacteria. Plates were incubated for $35^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 24 ± 2 hours before colonies with a gold metallic sheen were counted.

6.2.4 Propidium monoazide protocol PMA protocol

PMA is a DNA-intercalating dye that eliminates extracellular DNA as well as DNA from cells with damaged membranes from downstream qPCR processing. PMA stock solutions were created by dissolving 5 μL of 20 mM Propidium monoazide (PMA) (Biotium, Hayward, CA, USA) in 20% dimethyl sulfoxide (DSMO) (min 99.9%, Caledon Laboratory Chemicals, Georgetown, ON, Canada) and stored at 4°C and shielded from light in a sterile dark glass vial until used. A volume of 5 μL of the PMA stock solution was added to 1 mL of treated sample, mixed gently with a vortex mixer for 5 seconds and incubated in the dark at room temperature for 10 min. PMA-sample mixtures were

then exposed to a 150-watt halogen bulb for 10 min to bind the PMA to DNA. During light exposure the sample was placed on ice to keep the sample cool and gently mixed by hand every two minutes.

6.2.5 DNA extraction and quantification, and qPCR protocol

Genomic DNA was extracted using the Mo-bio PowerSoil DNA Isolation Kit (Carlsbad, CA, USA) which includes a bead beating step. Sample volumes of 1mL were centrifuged for 3 min at 10,000xg to form a pellet and the supernatant discarded. Samples were processed according to the instructions of the isolation kit with the exception that all incubation periods were increased from 5 to 10 minutes. DNA extracts were quantified using the Qubit 3.0 Fluorometer (Life Technologies, Eugene, OR) and stored at -20°C until used for qPCR analysis.

The qPCR protocol minimized false positives by using native Taq polymerase and included magnesium chloride (MgCl), DMSO (min 99.9%, Caledon Laboratory Chemicals, Georgetown, ON, Canada), and Bovine Serum Albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) which decreased the non-specific amplification due to humic acids in wastewater (Kibbee et al., 2013). The protocol amplified the *uidA* gene (see Table 6.1) of *E. coli*, which encodes for the β -glucuronidase enzyme and has been shown appear in 97.7 % of *E. coli* isolates from treated and raw water samples (Martins et al., 1993) and appears only once per genome (Taskin et al., 2011). Reactions were performed with: 5 μ L DNA template, 2.5 μ L 10xPCR Buffer (Invitrogen, Carlsbad, CA, USA), 1.25 μ L 50mM MgCl (Invitrogen, Carlsbad, CA, USA), 0.5 μ L 10 mM dNTPs (Invitrogen, Carlsbad, CA, USA), 1.25 μ L 5% DMSO min (99.9%, Caledon Laboratory Chemicals, Georgetown, ON,

Canada), 0.625 µL of 4 µM stock of forward primer, 0.625 µL of 4 µM stock of reverse primer, 1 µL 10 µg/µL BSA, 20X EvaGreen (Biotium Inc., Fremont, CA, USA), 0.5µL DNA Taq Polymerase (Native, Invitrogen, Carlsbad, CA, USA) and filled to 25µL with DNA-free water (Sigma-Aldrich, St. Louis, MO, USA). Cycling parameters were followed as denaturation at 95.0°C for 2 minutes, followed by 40 cycles of 95.0°C for 10 s, 61.4°C for 20s and 72.0°C for 15s. Subsequent to every qPCR run, a melt curve was performed for quality control purposes with increasing temperatures from 65.0°C to 95°C in 0.2°C increments with each increment lasting for 5s. Representative amplification and melt curve of a serial dilution of *E. coli* spiked wastewater was used to create a standard curve (see Chapters 3 and 4 for further details). Melt curve demonstrates a single peak at 84.6°C ± 0.2°C. All reactions were performed in triplicate and analyzed with the Bio-Rad CFX96-C1000 Real-Time PCR system and the Bio-Rad CFX Manager 3.1. The absolute quantification method was used throughout to the study to perform qPCR analysis and converted colony forming units (CFU) to calculated colony forming unit equivalents (CCE).

Table 6.1: PCR primers used in this study

Genetic Target	Primer	Sequence (5' → 3')	PCR Product Size (bp)	Reference
<i>uidA</i> gene	UAL 1939b	ATGGAATTCGCCGATTTTGC	187	(Heijnen and Medema, 2006a)
	UAL 2105b	ATTGTTTGCCTCCCTGCTGC		

6.2.6 Live/dead viability stain

BacLight® Live/Dead viability assay kit L13152 (Molecular Probes, Eugene OR) was used to visualize viable cells. Dyes from the kit were hydrated in 5 mL of deionized water and stored at 4°C in a sterilized dark glass container for up to one month. To perform the staining, samples were mixed in equi-volume amounts with the hydrated dye mixture, gently vortexing them for 5 seconds and finally incubated in the dark at room temperature for 10 minutes. Slides were viewed the Eclipse TE (Nikon, Montreal QC) microscope equipped with Intensilight C-HGFI fluorescent illuminator (Nikon, Montreal QC) and GFP-1, Cy3, GFPHQ filters (Nikon, Montreal QC). To obtain a representative image slides were visually scanned and preliminary cell counts were taken for a minimum of three fields of view. A picture of a representative field of view was taken with the Q Imaging Retina Exi Fast 1394 (Nikon, Montreal QC) camera and cell counts and image processing completed with NIS-Elements software (Nikon, Montreal QC).

6.2.7 Statistical analysis

One-way analysis of variance (ANOVA) was performed to determine if a significant difference exists between the means of two or more samples. The alternative hypothesis, that the means were different, was accepted if the *p*-value was less than the significance level (α) of 0.05. If accepted, the Tukey test was used post-hoc to further separate the means, using the same significance level (Currell and Dowman, 2005). Statistical analysis was performed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

6.3 Results and discussion

6.3.1 Culturable bacteria

The following experiments were designed to compare the performance of disinfection treatments using UV irradiation and ferrate(VI) when used both individually and in sequence. To do so, concentrations of *E. coli* (Figure 6.1 and Figure 6.2) and *C. perfringens* (Figure 6.3 and Figure 6.4) were evaluated after disinfection events with culture methods.

Figure 6.1-a demonstrates that when applied alone, UV irradiation reduced culturable *E. coli* from 7.66 Log CFU/mL to 2.43, 1.22, 1.77, and 1.10 Log CFU/mL with 10, 20, 40 and 60 mJ/cm². The UV inactivation curve shows the tailing effect where increasing fluence rates produce smaller gains in the reduction of bacteria which are typically associated with particles that are present in clumps (Kollu and Örmeci, 2012; Madge and Jensen, 2006; Qualls and Johnson, 1983). Water treatment plants have a typical fluence of 40 mJ/cm² while WWTP are typically designed for 60 mJ/cm². In this study, fluences above 20 mJ/cm² would allow regulatory standards to be met by reducing *E. coli* by 99.999% or to below 2 Log CFU/mL. Ferrate(VI) reduced culturable *E. coli* from 7.66 Log CFU/mL to 1.45, 1.31, 1.13 and 0.79 Log CFU/mL for concentrations of 9, 27, 36 and 54 mg/L as Fe(VI), respectively (Figure 6.1-b). Concentrations above 9 mg/L as Fe(VI) reduced *E. coli* by same quantities mentioned above and met regulatory limits of 2 Log CFU/mL. Statistical analysis was performed to determine the optimal concentration of ferrate(VI) to apply during sequential treatment. ANOVA testing demonstrated a significant difference among the means. A post-hoc Tuckey test was performed comparing the

effectiveness of 9 mg/L as Fe(VI) to that of 27, 36 and 54 mg/L as Fe(VI) and no observable difference was found ($p = 0.8171$ and $p = 0.2016$, respectively). Only the highest concentration of 54 mg/L as Fe(VI) was significantly different from 9mg/L as Fe(VI) ($p = 0.0054$). Based on these results, the concentration of 9 mg/L as Fe(VI) was determined to minimize the use of ferrate(VI) while maximizing the disinfection capacity and was selected for subsequent sequential disinfection experiments.

Figure 6.2 compares the effectiveness of individual and combined treatments of UV fluence of 40 mJ/cm² and 9 mg/L as Fe(VI) of ferrate(VI). The combined UV irradiation and ferrate(VI) treatment reduced *E. coli* concentration to 0.6 Log CFU/mL and was found to be significantly different from individual treatments of UV ($p = 0.0025$) and ferrate(VI) ($p = 0.0032$). When compared to UV fluence of 40 mJ/cm², the combined treatment achieved an additional 0.9 Log reduction and had better performance than 60 mJ/cm². The increased performance of the combined treatment may also eliminate the tailing effect often observed with UV irradiation.

Figure 6.1: Inactivation of *E. coli* in secondary wastewater subsequent to (a) UV irradiation at UV fluences of 0, 10, 20, 40 and 60 mJ/cm² (b) ferrate(VI) at concentrations of 0, 9, 27, 36 and 54 mg/L as Fe(VI)

Figure 6.2: Inactivation of *E. coli* in secondary wastewater subsequent to UV fluence of 40 mJ/cm^2 , 9 mg/L Fe(VI) and sequential disinfection of UV and ferrate(VI), and ferrate(VI) and UV

Culture methods are the standard methods used to assess the effectiveness of wastewater disinfection. Typically, enteric non-pathogenic indicator organisms are cultured on selective media (World Health Organization, 2003). However, the absence of indicator organisms does not confirm the absence of other pathogenic and more resistant organisms, such as enteric viruses or protozoans (e.g. *Giardia* cysts and *Cryptosporidium* oocysts). For these reasons, *C. perfringens* has been selected as a secondary organism to evaluate in this study. *C. perfringens* is an enteric bacteria that has been used as an indicator for sewage contamination because they can survive for long periods of time (Hill et al., 1993; Skanavis and Yanko, 2001). Similar to *E. coli*, *C. perfringens* are rod-shaped bacteria with readily available culture media and laboratory protocols. Unlike *E. coli*, *C. perfringens* are gram-positive, anaerobic, and spore-forming. Additionally, removal of *C. perfringens* has been shown to be correlated to the removal

of enteric viruses, *Giardia* cysts, and *Cryptosporidium* oocysts (Payment and Franco, 1993). Other studies have also suggested the use of *C. perfringens* as a surrogate for *Cryptosporidium* (Abia et al., 2015; Fujioka and Shizumura, 1985; Harwood et al., 2005; Venczel et al., 1997).

Figure 6.3 compares the effect of the individual treatments of UV irradiation and ferrate(VI) on the culturability of *C. perfringens*. In this experiment, indigenous *C. perfringens* in wastewater were studied. Results of the effects of UV irradiation, shown in Figure 6.3-a, demonstrate that *C. perfringens* were reduced from 3 Log CFU/100mL to 2.72, 2.33, 1.95 and 1.44 Log CFU/100mL with fluences of 10, 20, 40 and 60 mJ/cm² with a strongly linear trend ($R^2 = 0.96$). The most common UV fluences of 40 mJ/cm² and 60 mJ/cm² achieved 95.5% and 98.6 % reduction, respectively. *C. perfringens* are more resistant to UV than other indicators organism such as fecal coliforms and *E. coli*, as reflected in the lower removal percentages, and, for this reason, have been suggested as a conservative indicator (Gehr et al., 2003). The tailing effect is also notably absent from Figure 6.3-a, as indicated by a near-linear relationship ($R^2 = 0.96$). A similar result was observed by Gehr et al. (2003) with *C. perfringens* and UV as well as by Qualls and Johnson (1983) who worked *Bacillus Subtilis*, an aerobic spore-forming bacteria. The tailing effect is typically attributed with organisms being entrapped in particles as well as with particle shielding. Gehr et al. (2003) reasoned that the risk of shielding was minimized due to long exposure times of 10-15 minutes and constant mixing of the sample, which was also the case during experiments presented here. Therefore, leaving the reason for the lack of tailing to be attributed to *C. perfringens* having a lower

association to particle entrapment than other bacteria (Gehr et al. 2003). While this is possible, it is also possible that due to *C. perfringens*' resistance to UV, that the tailing effect would only be expected to be observed at higher fluences beyond what were used in this study. In addition, spore-forming bacteria may have different deactivation mechanism than non-spore formers and therefore exhibit different UV deactivation curves.

Figure 6.3-b shows the effectiveness of ferrate(VI) on the reduction of indigenous *C. perfringens* in wastewater. For concentrations of 3, 6, 9 and 15 mg/L Fe(VI) *C. perfringens* concentrations were measured to be 3.30, 3.15, 3.30 and 2.59 Log CFU/100 mL from starting concentrations of 3.6 Log CFU/100 mL. A linear decreasing trend ($R^2 = 0.46$) of *C. perfringens* was observed with increasing concentrations of ferrate(VI). *C. perfringens* have been shown to be more resistant to chemical disinfection such as chlorine (Fujioka and Shizumura, 1985; Venczel et al., 1997), ozone (Gehr et al., 2003), and ferrate(VI) (Kim et al., 2005). Figure 6.4 shows the effect of the sequential treatment of UV irradiation and ferrate(VI) on the concentration of *C. perfringens* where an additional 0.7 Log reduction was observed. However, this reduction was not found to be significant ($p = 0.112$) when compared to the use of UV irradiation alone. It is important to note that due to the low concentration as well as the variability of the culture method the error associated with these results were higher than expected and played a role to the outcome of the statistical analysis.

Results presented here demonstrate that when combined, treatments of UV and ferrate(VI) offer increased reduction in culturable *E. coli* and *C. perfringens*. In addition, the tailing effect often seen with UV disinfection can also be overcome.

Figure 6.3: Inactivation of *C. perfringens* in secondary wastewater subsequent to (a) UV fluences of 0, 10, 20, 40 and 60 mJ/cm² (b) ferrate concentrations of 0, 3, 6, 9, 18 and 30 mg/L as Fe(VI)

Figure 6.4: Inactivation of *C. perfringens* in secondary wastewater subsequent to UV fluence of 40 mJ/cm², 9 mg/L Fe(VI) and sequential disinfection of UV irradiation and ferrate(VI), and ferrate(VI) and UV irradiation

6.3.2 Viable but nonculturable bacteria

The purpose of the following experiment was to evaluate the VBNC-state of *E. coli* following individual and combined treatment of UV irradiation with a fluence of 40 mJ/cm² and ferrate(VI) with a concentration of 9 mg/L as Fe(VI). The VBNC state in bacteria is a pseudo-dormant state where bacteria are no longer able to be cultured but remain viable and can retain metabolic activity and virulence (K L Cook and Bolster, 2007; Kana et al., 2008; Oliver, 2010; Oliver et al., 1995b; Sun et al., 2008). Environmental stress such as low nutrients (K L Cook and Bolster, 2007), low temperatures (Maalej et al., 2004; Wong et al., 2004), low oxygen (Kana et al., 2008), and, most importantly for WWTPs, oxidative stress following disinfection (Noor et al., 2009; Oliver et al., 2005; Servais et al., 2009) can all induce the VBNC-state. VBNC-

bacteria may also be tolerant to antibiotics (Ayrapetyan et al., 2015; Nowakowska and Oliver, 2013), which have been found in wastewater, and may contribute to the spread of antibiotic resistant genes (Marti et al., 2013). Recent research has shown that enteric VBNC bacteria could regain culturability when co-cultured with eukaryotic cells, which can have implications for gastrointestinal illnesses (Senoh et al., 2012). It is therefore important for WWTPs to consider the detection and control of VBNC bacteria.

Figure 6.5 presents and compares results of the culture method, as well as molecular methods qPCR, and PMA-qPCR. In the first series of columns, change in the culturability of *E. coli* is demonstrated. Initially, culturable *E. coli* were measured at 7.61 Log CFU/mL and were decreased to 1.49 Log CFU/mL with 40 mJ/cm², 2.85 Log CFU/mL with 9 mg/L as Fe(VI) and to 0.6 Log CFU/mL when treatments were combined. The second series of columns show results of the qPCR method. When used alone, qPCR provides the total quantity of DNA from *E. coli* that was able to be extracted from a sample. However, DNA is a conservative molecule and can survive long after the death of a cell both intracellularly and extracellularly. Thus, the use of qPCR alone does not provide any information on the viability of cells. Results of qPCR were used to confirm that a consistent quantity DNA, between 7.33 and 7.50 Log CCE/mL, was being extracted from all samples regardless of treatment. No statistical difference was found between the quantity of DNA extracted with qPCR ($p = 0.8290$).

The third series of columns of Figure 6.5, presents results of the PMA-qPCR method. PMA is a DNA-intercalating dye that eliminates extracellular DNA as well as DNA from

membrane-damaged cells from downstream qPCR processing. With this method, the integrity of the cellular membrane is used as an indication of cell viability. This method is suitable to detect viable cells following chemical oxidation but may be problematic for use with UV irradiation. That is because UV irradiation works by creating pyrimidine dimers in the nucleic acid of the cell which renders a cell unable to replicate. These changes to the DNA do not directly affect the integrity of the cell wall. Results of the PMA-qPCR method demonstrate that 7.48 Log CCE/mL *E. coli* remained viable after a UV fluence of 40 mJ/cm² and 7.52 Log CCE/mL *E. coli* remained viable after a ferrate(VI) concentration of 9 mJ/cm². In other words, both individual treatments provided an approximate log reduction of 0.93. Compared to results from the first series of columns (Figure 6.5), there is approximately 6.7 Log CFU/mL that remain in a VBNC state following individual disinfection with UV irradiation and ferrate(VI). Other studies have shown that oxidative stress due to chemical disinfection can induce the VBNC-state (Noor et al., 2009; Oliver et al., 2005; Servais et al., 2009). For example, Oliver et al. (2005) studied different states of *E. coli* and *Salmonella typhimurium* after exposure to chlorine and found that 4 Log cells/mL of the initial 6 Log cells/mL survived in a VBNC state as determined by the direct viable count (DVC). As previously discussed, UV irradiation does not affect membrane integrity and reductions in the quantity of DNA measured may be attributed to changes in the DNA sequence created by the formation of pyrimidine dimers. However, changes to the DNA sequences still indicate that UV treatment was effective. Similar observations have been made by other researchers who have used these changes to the DNA sequence to measure the extent of UV-

induced damage to cells. For example, Van Houten et al., (2000) used differences in qPCR results to examine changes in long fragments of DNA (10^3 - 10^4 bp) that occurred in human genes exposed to 100 mJ/cm^2 UV. Eischeid et al. (2009) also used qPCR in a similar way to study UV-induced lesions of adenovirus DNA following low and medium pressure UV. In a separate study, Süß et al. (2009) compared different lengths of DNA fragments to monitor the repair of UV-induced DNA-damage of bacterial communities of wastewater such as *Pseudomonas aeruginosa* and *Enterococcus faecium*.

The combined treatment of a UV fluence of 40 mJ/cm^2 and ferrate(VI) concentration of 9 mg/L as Fe(VI) achieved the greatest reduction in viable bacteria where 6.77 Log CCE/mL was observed, which translates to a 1.66 Log reduction when compared to the initial wastewater sample and an additional 0.7 Log reduction when compared to individual treatments. Combined treatment was the most effective at reducing the quantity of viable *E. coli*. However, a large amount still remains.

To complement the PMA-qPCR method, the live/dead viability stain was used to provide a visualisation of viable and non-viable cells following treatment. The viability stain utilizes two nucleic acid stains that distinguish between live and dead cells in a similar way as the PMA-qPCR technique. That is, membrane integrity is used as the indicator of cell viability. The SYTO-9 stains all nucleic acid to fluoresce green, and propidium iodide penetrates cells with damaged membranes labeling these cells red. Cells that fluoresce red are indicative of a dead or non-viable cell, and a green fluorescing cell indicates a viable cell. It is important to note that the live/dead staining is not organism specific. In

these experiments, *E. coli* was spiked into non-autoclaved wastewater which contains a heterogeneous population of microorganisms. Results presented here include other bacteria that may be found in wastewater.

Results of the live/dead analysis are presented in Figure 6.6 and representative images in Figure 6.7. Comparing results of the initial wastewater to those following UV fluence of 40 mJ/cm², there is little difference between both the total number of cells as well as the percent live and dead cells. UV does not affect the permeability of the cellular membrane, and therefore little change was to be expected. Ferrate(VI), on the other hand, is an oxidative treatment and as such a greater degree of damage to the cell wall was expected. A decrease to the total number of cells and as well as an increase in the percent of dead cells (to 32%) is observed following ferrate treatment of 9 mg/L Fe(VI). A reduction in the total number of cells indicates that cells have been completely lysed and are no longer countable with the live/dead viability stain. An increase in the percent of dead cells indicates that a greater proportion of cells had damaged membranes and are non-viable. The greatest reduction of cells was observed with the combined treatment UV fluence of 40 mJ/cm² and 9 mg/L Fe(VI), which also had 4% dead cells. The trends described by the live/dead assay are in line with those observed with the PMA-qPCR treatment and help to validate the results.

Results presented here are that bacteria in the VBNC-state are common and in abundance following disinfection treatments of wastewater despite culturable methods indicating otherwise.

Figure 6.5: inactivation of *E. coli* in wastewater with starting concentration of 7.61 Log CFU/mL subsequent to 9 mg/L Fe, 40 mJ/cm² and combined use of 40 mJ/cm² and 9 mg/L Fe(VI) as measured by culture methods, qPCR, and PMA-qPCR (n = 3)

Figure 6.6: Number of live and dead cells as determined with the live/dead viability stain of secondary wastewater spiked with *E. coli* and treated with treatment UV fluence of 40 mJ/cm², 9 mg/L Fe(VI) and the combined treatment of UV fluence of 40 mJ/cm² and 9 mg/L Fe(VI) (n = 3)

Figure 6.7: Images of the live/dead viability stain from a representative field of view of secondary wastewater spiked with E.coli and treated with treatment UV fluence of 40 mJ/cm², 9 mg/L Fe(VI) and the combined treatment of UV fluence of 40 mJ/cm² and 9 mg/L Fe(VI) (n = 3)

6.3.3 Regrowth, reactivation, and resuscitation

The long-term effectiveness of treatments and regrowth potential was studied in this section and results are presented in Figure 6.8. The study was completed by monitoring changes in the quantities of culturable indigenous coliform bacteria for 48 hours after disinfection of wastewater with different treatments. Indigenous coliform bacteria were selected, instead of *E. coli*, because they allow for a broader and more realistic perspective on the disinfection efficiency of treatments. In addition, un-seeded and unmodified wastewater samples were used for these experiments to allow for an understanding of the in situ response of coliform bacteria. Treatments examined include wastewater as it enters the secondary clarifier, wastewater as it exits disinfection with sodium hypochlorite, following UV fluence of 40 mJ/cm², following 9 mg/L as Fe(VI), and following the sequential treatment of UV fluence of 40 mJ/cm² UV and 9 mg/L Fe(VI). The time step 0 hours indicates that measurements were performed immediately following treatment.

To study the long-term effectiveness of disinfection, the terms regrowth, reactivation and resuscitation are commonly used and will be discussed here. Regrowth is defined as the reproduction of healthy microorganisms that survive disinfection and rely on the availability of nutrients required for replication (Kollu and Örmeci 2014). From a laboratory perspective, it is difficult to provide an experimental matrix free of nutrients as intracellular material from damaged or lysed cells can provide nutrients required for regrowth (Dukan et al., 1997; Kollu and Örmeci, 2014). It has been estimated that 50 dead cells are required to sustain the growth of one cell (Postgate and Hunder, 1962).

Reactivation is the repair of sub-lethal damage of a cell following UV irradiation where the ability to reproduce is regained. It is often further divided into light and dark repair. To minimize the effects of light repair, samples in this series of experiments were kept in the dark (Malley, 2004). The term resuscitation is used to indicate a VBNC cell that regains culturability (Dukan et al., 1997; Oliver et al., 1995b). It is marked by a recovery time less than what would be expected by regrowth because repair and replication are not required to see an increase in population density as is the case with regrowth (replication only) and reactivation (repair and regrowth). In the case of resuscitation, cells that were in a VBNC-state with low metabolic activity regain the capacity to be cultured. All three terms will be used below to discuss the results.

The change of the culturability of coliform bacteria in wastewater with time and without any treatment is presented in Figure 6.8-a. These results are used to compare the response of the coliform population after sodium hypochlorite (6.8-b), UV (6.8-c), ferrate(VI) (6.8-d), and the combined use of UV and ferrate(VI) (6.8-e) treatments. ANOVA testing was performed on the data and demonstrates that a statistically significant decrease in the concentration of culturable coliform bacteria occurred within the data set of the baseline case ($p = 0.0082$). Post-hoc Tukey test compared the initial (0 hours) concentration of coliform bacteria to that at each time step. Results of this test indicate that a difference of the mean from the initial time step was statistically different at 48 hours ($p = 0.0067$). A similar statistical analysis is performed for all treatments, results of which are shown in Table 6.2. In a laboratory setting with favorable conditions (e.g. pH, nutrients, culture media, temperature, oxygen

requirements, etc.) the generation time of coliform bacteria can typically range from 15-20 minutes. In the intestinal track of humans, the generation time can be as long as 12-24 hours. Results of experiments presented here were conducted at room temperature in low-nutrient wastewater and a long generation time was expected. Results show that in a wastewater matrix with natural cell die off no regrowth occurred within the 48 hour test period. Following treatment with UV and ferrate(VI), solubilisation of organic compounds could contribute to an increase of available nutrients.

Similar monitoring and statistical analysis were performed for samples taken following secondary treatment following sodium hypochlorite (see Figure 6.8-b). ANOVA testing showed that a significant difference within the data occurred ($p < 0.0001$) and post-hoc Tukey test indicated that the difference occurred at 6 hours ($p = 0.00304$). Chlorine disinfection provided an initial 1.72 Log-reduction in culturable coliform bacteria immediately after treatment, but gains were reduced ultimately reduced to 0.57 Log-reduction after 48 hours. Previous research (see Chapter 4) has shown that a significant proportion of *E. coli* enter the VBNC-state following the oxidative stress of sodium hypochlorite. As an extension, it would be expected that a large proportion of coliform bacteria could also enter the VBNC-state making resuscitation a possible explanation for the increase in population observed. In addition, sodium hypochlorite works by oxidizing cellular components which induce leaching of intracellular material allowing for the possibility of regrowth of surviving cells to also occur (Cho et al., 2010; Venkobachar et al., 1977). Dukan et al. (1997) studied regrowth and reactivation of *E. coli* in PBS buffer following treatment with sodium hypochlorite. The authors showed that cells damaged

with sodium hypochlorite were able to recover in PBS due to nutrients provided by damaged cells, which further supports the argument for regrowth. However, the study also found that stressed cells grew at a significantly higher rate than unstressed cells and growth rates remained elevated for up to 15 generations. This apparent increase in growth rate could also be explained by cells exiting the VBNC-state via resuscitation.

Changes in culturability following UV irradiation are presented in Figure 6.8-c. A significant increase in the concentration of culturable coliform bacteria was observed within the dataset ($p < 0.0001$) and the increase was significant at 18 hours ($p = 0.009$) and beyond. UV irradiation is a physical disinfection method that forms primer dimers in the nucleic acid of cells, leaving the cell membrane and other internal components intact (Cho et al., 2010). The release of intracellular material, and consequently nutrients, due to lysed cells is therefore not as extensive as with chemical disinfectants. In addition, due to the working mechanism of UV irradiation, the identification of bacteria in the VBNC-state was not conclusive (see Chapter 4) and therefore hypothesizing that no resuscitation occurred. The increase in population observed at 18 hours can therefore primarily be attributed to reactivation (due to dark repair) and regrowth (due to the availability of nutrients in wastewater).

Results following ferrate(VI) treatments are shown in Figure 6.8-d where a 1.35 Log-reduction of bacteria was observed, and no significant increase in the population of culturable coliform bacteria was observed for the entire 48 hour period ($p = 0.6495$). Compared to sodium hypochlorite, ferrate(VI) is a stronger oxidant, which may explain

the difference in the observed response. Stronger oxidants cause greater damage to the surface structures of cells while weaker oxidants are able to permeate the cell membrane to have a greater effect on internal cell components (Cho et al., 2010; Venkobachar et al., 1977). If the integrity of the cell membrane is lost, then a lower degree of reactivation would be expected. Additionally, previous work (see Chapter 4) showed that ferrate(VI) was more effective at reducing VBNC bacteria. By extension a lower degree of resuscitation would be expected. Although no regrowth or resuscitation was observed during the time period of the experiment, it is possible that cells damaged by ferrate(VI) need additional time for recovery.

Results of the combined treatment of UV and ferrate(VI) is presented in Figure 6.8-e and a 2.83 Log-reduction in culturable coliform bacteria is observed. Similar to treatment with UV, growth was statistically significant at 18 hours ($p = 0.0478$). The use of UV in the combined treatment implies that reactivation is in part accountable for the increases observed. Regrowth and resuscitation of cells is also possible, but since these rates were low when ferrate(VI) was used alone, they are also expected to be low when treatments of UV and ferrate(VI) are used together.

Monitoring the growth of coliform bacteria for 48 hours after treatment demonstrated that regrowth and resuscitation were observed the soonest after treatment with sodium hypochlorite suggesting that low strength oxidants allow for greater risk for changes in the bacteria population to be observed. Regrowth and reactivation were observed with UV as was expected. No significant increases in culturable coliform bacteria were

observed with ferrate(VI) suggesting that stronger oxidants that have a greater effect on the cell wall may also prevent reactivation of damaged cells and resuscitation of VBNC cells. The combined treatment of UV and ferrate(VI) showed a similar trend to that of UV alone suggesting that reactivation mechanism were primarily accountable for increases in the coliform population. However, regrowth and resuscitation are also possible.

(a) Secondary Influent

(b) Secondary Effluent (sodium hypochlorite)

Figure 6.8: Inactivation of coliform bacteria after 0, 6, 18, 24 and 48 hours (a) secondary effluent wastewater (before chlorination) (b) final effluent wastewater (after chlorination) (c) UV fluence of 40 mJ/cm² (d) 9 mg/L as Fe(VI) (e) sequential treatment of UV fluence of 40mJ/cm² and 9 mg/L Fe(VI) (n = 3)

Table 6.2: Results of Statistical Analysis from Regrowth Study

	Treatment				
	Wastewater	Chlorine	UV	Fe	UV + Fe
Results from Initial the ANOVA Test					
<i>p</i> value	0.0082 (S*)	<0.0001 (S)	<0.0001 (S)	0.6459 (NS†)	<0.0001 (S)
Post-Hoc Tukey Test					
Adjusted <i>p</i> value for 0 vs. 6 hours	0.9520 (NS)	0.0304 (S)	0.2898 (NS)	N/A‡	0.6646 (NS)
Adjusted <i>p</i> value for 0 vs. 18 hours	0.2099 (NS)	<0.0001 (S)	0.0009 (S)	N/A	0.0478 (S)
Adjusted <i>p</i> value for 0 vs. 24 hours	0.2276 (NS)	<0.0001 (S)	0.0004 (S)	N/A	0.0478 (S)
Adjusted <i>p</i> value for 0 vs. 48 hours	0.0067 (S)	<0.0001 (S)	<0.0001 (S)	N/A	<0.0001 (S)

* S = statistically significant

‡ NS = not statistically significant

‡ N/A = not appropriate to apply further statistical analysis (i.e. the Tukey test)

6.4 Conclusion

The purpose of this study was to investigate the use of UV and ferrate(VI) both alone and in combination for wastewater disinfection. When used alone or in combination, the study found UV and ferrate(VI) were effective at reducing culturable *E. coli* to below regulatory standards. The combined treatment increased reductions, which allowed for the tailing effect (often seen with UV) to be overcome. The combined use of ferrate(VI) and UV was not as effective at reducing culturable *C. perfringens*. No additional gains were achieved when compared to the use of UV alone. However, culture methods used to evaluate *C. perfringens* were associated with a higher degree of uncertainty.

Molecular methods were also used to determine VBNC *E. coli* following treatment. Of the initial 7.6 Log CCE/mL of *E. coli* in wastewater, 6.7 Log CCE/mL remained viable after treatment with ferrate(VI) and 5.6 Log CCE/mL remained viable after the combined use of UV and ferrate(VI) when culturability was reduced to be low below 2 CFU/mL. The combined use of UV and ferrate(VI) was more effective at reducing viable *E. coli* compared to the use of ferrate(VI) alone.

Changes to the culturability of indigenous coliform bacteria were monitored for 48 hours to provide insight into potential regrowth, reactivation, and resuscitation of bacteria. The study demonstrated that regrowth and resuscitation were observed the soonest after treatment with sodium hypochlorite. Regrowth and reactivation were observed with UV at 18 hours. No significant increases in culturable coliform bacteria was observed with ferrate(VI) suggesting that stronger oxidants that have a greater effect on the cell wall may prevent the reactivation of damaged cells and resuscitation of VBNC cells. The combined treatment showed a similar trend to that observed with UV indicating that reactivation and regrowth after 18 hours.

6.6 References

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Chapter 7

7 Inactivation and regrowth of culturable and viable but nonculturable (VBNC) bacteria after combined use of ultraviolet light and peracetic acid for wastewater disinfection

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Abstract

This study investigated the response of *Escherichia coli* and *Bacillus subtilis* following wastewater disinfection with ultraviolet light (UV) and peracetic acid (PAA). The culturable and viable, but nonculturable (VBNC) state of indicator organism *E. coli* were studied, as was their potential to regain culturability, without nutrient supplementation, for a period of 48 hours after treatment. The VBNC state was examined using molecular methods such as the live/dead viability stain as well as a method that combined the use of propidium monoazide (PMA) and quantitative polymerase chain reaction (qPCR). The behavior of spore-forming bacteria *B. subtilis* was also included to examine microorganism that is more resistant to disinfection. Results of the study showed that UV supplemented with PAA was equally (in the case of *B. subtilis*) or was more (in the case of *E. coli*) effective than using either treatment alone. The PMA-qPCR method measured viable *E. coli* and highlighted that a large quantity *E. coli* are escaping detection when measured with culture methods. The combined use of UV and PAA

further reduced the quantity of viable cells escaping treatment compared to when each treatment was used alone. However, many viable cells still remained. The culturability of *E. coli* was monitored for up to 48 hours and the bacteria re-gained culturability after a lag period between 6 and 24 hours without additional nutrient supplementation. Acetic acid present in the equilibrium mixture of PAA was found not to be a nutrient source contributing to regrowth. The contribution of the PAA-oxidized organic matter remains to be confirmed as a contributing factor. Supplementing UV with PAA is an attractive disinfection treatment that both eliminates the risk of chlorine-based disinfection byproducts (DBPs) and shows increased disinfection levels compared to when either UV or PAA was used alone. This research highlights the need to expand the type of indicator organisms examined as well as to incorporate the use of molecular methods to examine the viability of bacteria. In addition, longer regrowth periods should be studied for a better understanding of what occurs once wastewater is discharged into the environment.

Keywords: Wastewater, disinfection, qPCR, PMA-qPCR, VBNC, UV, PAA, peracetic acid, VBNC

7.1 Introduction

Chlorine is the most widely disinfectant for wastewater worldwide despite its potential to form harmful disinfection byproducts (DBPs) and that chlorine residuals have been shown to have toxicological effects (Liviak et al., 2010; Monarca et al., 2000; Plewa et al., 2012; Watson et al., 2012). As an alternative, ultraviolet light (UV) is a physical

disinfection method that leaves no chemical residual and demonstrates an effectiveness against chlorine-resistant protozoa such as *Cryptosporidium parvum* oocysts and *Giardia lamblia* (Bukhari et al., 1999; Dell’Erba et al., 2007; Liberti et al., 2003; Linden et al., 2001). UV creates pyrimidine dimers in the DNA of microorganisms rendering them incapable of replication and, in turn, incapable of causing infection. UV relies on the transmission of light to suspended microorganisms; changes to wastewater flows or concentration of suspended solids can make the maintenance of consistent effluent quality a challenge for wastewater treatment plants (WWTP) (Caron et al., 2007; Li et al., 2009).

Recent research has examined the combined or sequential use of two disinfectants as a means of improving the microbiological quality of effluent wastewater (Cho et al., 2011, 2006b; Meunier et al., 2006; Murphy et al., 2008; Rennecker et al., 2000; Shin and Lee, 2010; Wang et al., 2012; Young and Setlow, 2003). For example, spore-forming bacteria, such as *Bacillus subtilis*, are more difficult to inactivate and can enter a protective spore state when stressed, as is the case when exposed to chemical oxidants. However, research has shown that the sequential use of chlorine dioxide and free chlorine can further reduce concentrations of *B. subtilis* (Cho et al., 2006a). Beneficial effects have also been observed when chlorine was combined with ozone (Rennecker et al., 2000) or UV (Murphy et al., 2008). The combined use of UV with chlorine-free chemical agents has also been examined. This combination has additional advantages of minimizing the use of chemicals, decreasing the formation of chlorine DBPs and offering WWTPs increased operational flexibility. Disinfection combinations that were investigated

included the use of UV with hydrogen peroxide (HP) (Cho et al., 2011; Koivunen and Heinonen-Tanski, 2005; Vankerckhoven et al., 2011), with iodine (Pennell et al., 2008), with ozone (Jung et al., 2008), and with peracetic acid (PAA) (Koivunen and Heinonen-Tanski, 2005).

PAA is the peroxide of acetic acid and has been shown to be an effective bactericide and virucide (Lazarova et al., 1998; Rajala-Mustonen et al., 1997; Wagner et al., 2002). As a disinfectant, PAA requires short contact times and is effective over a pH range of 1-10, and a temperature range of 0-100°C (Lazarova et al., 1998; Rajala-Mustonen et al., 1997; Wagner et al., 2002). It has a similar oxidation-reduction potential to chlorine, and its disinfection mechanism has also been suggested to be similar (Rutala and Weber, 2008). Subsequent to oxidation, the reaction products of PAA include acetic acid (CH_3COOH), oxygen (O_2), methane (CH_4), carbon dioxide (CO_2), and water (H_2O) (Metcalf & Eddy, 2003). In addition, no evidence of aldehydes or halogenated phenols, two common groups of chlorine DBP were found in secondary wastewater treated with PAA (Dell'Erba et al., 2007). The main DBP of PAA were shown to be carboxylic acids; a functional group that contains acetic acid, one of the reaction products mentioned above (Monarca et al., 2002a, 2002b). The use of PAA as a wastewater disinfectant has been associated with increases in biodegradable organic carbon (Lazarova et al., 1998) as well as increases in COD (Collivignarelli et al., 2000).

When PAA is combined with UV, mixed oxidative species such as single oxygen, ozone, and hydroxyl radicals are formed (Caretto and Lubello, 2003; Koivunen and Heinonen-

Tanski, 2005; Vankerckhoven et al., 2011). Koivunen and Heinonen-Tanski (2005) observed an increase in disinfection efficiency when PAA was followed by UV for the disinfection of wastewater. Caretti and Lubello (2003) examined the use of PAA added before and after UV and found that both regimes offered increased efficiency. However, gains were greater when PAA was added upstream of the UV reactor suggesting increased efficiency was due to the formation of previously mentioned oxidative species. In a separate study, an additional 0.8 Log-reduction in bacteria was observed when 2.3 mg/L PAA was added to pilot-scale UV disinfection treatment system in Belgium (Vankerckhoven et al., 2011).

The purpose of this study was to examine the combined use of PAA and UV for the disinfection of secondary effluent wastewater. The short-term (immediately following disinfection) and long-term (48 hours after disinfection) culturability of indicator organism *E. coli* as well as spore-forming *B. subtilis* were studied. In addition, viable *E. coli* was quantified using PMA-qPCR, a method that has been shown to be effective at excluding DNA from nonviable cells from qPCR processing (Kibbee and Örmeci, 2017). The use of the PMA-qPCR has been successfully applied to a variety of matrices including saline buffers (Nocker et al., 2007; Nocker and Camper, 2006; Wahman et al., 2009; Zhang et al., 2015), wastewater (Bae and Wuertz, 2009; Kibbee and Örmeci, 2017; Varma et al., 2009), and biosolids (van Frankenhuyzen et al., 2013). In addition, the regrowth potential of *E. coli* was investigated, as was the contributions of acetic acid, which is both a reaction product of PAA oxidation and is added as part of the PAA mixture, to regrowth.

7.2 Materials and methods

7.2.1 Peracetic acid

VigorOx WWTII (FMC Corporation, Philadelphia, PA) PAA mixture was used directly without dilution throughout the course of the study. The formulation is a commercial mixture containing 15% PAA, 23% HP, 16% acetic acid, 1% sulfuric acid, and 45% water and has a specific gravity of 1.16g/mL at 20°C. Experiments were performed with a bench-scale jar tester, which provided continuous mixing at 70 rpm. All experiments were conducted in sterile glass beakers that were wrapped in aluminum foil to protect samples from light exposure. A contact time of 15 minutes was allowed for all experiments except when the UV irradiation time exceeded 15 minutes; see Section 7.2.3 for further details. PAA was quenched in using 2.0% (w/v) solution of autoclaved sodium thiosulfate (Fisher Scientific, New Jersey NY) with a 2 minute reaction time followed by 50 mg/L catalase which was dissolved in 50 mM potassium phosphate buffer (pH 7) (Sigma, St. Louis MO). Catalase is an antioxidant used to scavenge residual HP (Cho et al., 2006a; Wagner et al., 2002). The sterility of the catalase solution was assured daily by testing 10µL and 100µL by membrane filtration on EC Medium with MUG (Becton Dickenson and Company, Sparks, MD, USA). The effectiveness of the quenching protocol was confirmed by colorimetric method in which 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfuric acid (ABTS) and horseradish peroxidase (HRP) react with PAA (at pH of 6.0) to form an ABTS radical with peak absorbance 405 nm, which was measured with a UV-Visible spectrophotometer (Varian Model Cary 100BIO) (Wagner et al., 2002).

7.2.2 Ultraviolet light

A bench-scale collimated-beam apparatus with low-pressure UV lamps was used throughout the study. Procedures and germicidal fluence calculations followed those for suspension depth less than 2 cm (Bolton and Linden, 2003). A sample volume of 150 mL was placed in a tall-walled sterile glass petri dish and placed on a mixing plate that served as the irradiation stage and allowed for continuous mixing. The apparatus was equipped with four mercury LP-UV lamps (Phillips UV-C germicidal lamps), which provided nearly monochromatic UV light at 253.7 nm. Two apertures were placed below the lamps to focus the beam. The internal surfaces of the apparatus were painted with a matt black paint to assure little to no reflectivity. Prior to every experiment, sample absorption at 254 nm was measured using a UV-Visible spectrophotometer and 1-cm quartz cuvette. The intensity of the UV light required to measure fluence times was measured using an International Light NIS Traceable Radiometer/Photometer Model IL 1399A radiometer. Average light intensity measured throughout the course of experiments was $0.311 \pm 0.012 \text{ mW/cm}^2$.

7.2.3 Combined use of ultraviolet light and peracetic acid

Combined disinfection experiments allowed PAA contact time of 15 minutes and UV irradiation to occur simultaneously. To accomplish this, samples were placed in the UV reactor where PAA was added before the reactor was closed and turned on. If the UV irradiation time was less than 15 minutes, lamps were turned off after the irradiation time elapsed but samples remained in the reactor until the 15 minute PAA contact time

was completed. In the cases where UV irradiation times exceeded 15 minutes, PAA contact times were extended to meet irradiation times.

7.2.4 Bacterial strains

7.2.4.1 *Escherichia coli*

E. coli K-12 strain 3000-141 (ATCC® 19853, Cedarlane Laboratories, ON, Canada) was used throughout the study. Cultures were inoculated from frozen stock cultures in Bacto™ Tryptic Soy Broth Soybean-Casein Digest Medium (TSB) (Becton Dickinson and Company, MD, USA) in a ratio of 1:100 and incubated for 18 hours at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Pure cultures were washed 3 times by centrifuging samples for 5 mins at 8,000xg to form a pellet, discarding the supernatant and resuspending the pellet with phosphate buffered solution (PBS) (Eaton et al., 2005).

7.2.4.2 *Bacillus subtilis*

Bacillus subtilis (ATCC® 19659 Cedarlane Laboratories, ON, Canada) was revived overnight at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ by streaking frozen stock culture on Bacto™ Tryptic Soy Broth Soybean-Casein Digest Medium (TSB) by BD Bioscience (Sparks MD) to which 1.5% (w/v) agar (Agar Laboratory Grade ARG003.500. Bishop, Burlington, ON, Canada) was added. A spore stock solution was made by streaking overnight culture onto TSB with added agar for 26 days at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ after which the formation of spores was confirmed by phase contrast microscopy (no image). Colonies were washed with PBS and heated to 70°C for 20 minutes to inactivate vegetative cells. The heat-treated suspension was washed 8-times centrifuging samples for 10 minutes at 10,000xg, discarding the

supernatant and suspending in sterilized deionized water to remove vegetative cells (Kornberg and Spudich, 1969). Malachite green was used to stain and enumerate spores, see Figure 7.1. The final suspension had a cell concentration of 3.7×10^8 colony forming units (CFU)/mL *B. subtilis* containing 65% spores and stored at 4°C for up to four weeks.

Figure 7.1: *B. subtilis* stained with malachite green imaged with Nikon Eclipse TE oil immersion light microscope at 100x magnification (a) mixed vegetative and spore suspension (b) mixed vegetative and spore suspension subsequent to washing and heat treatments, this suspension was used in experiments.

7.2.4.3 *Pseudomonas aeruginosa*

Two strains of *P. aeruginosa* were used: *P. aeruginosa* ATCC® 19853 and *P. aeruginosa* PAO1H2O. The latter strain was isolated from wastewater and confirmed by BLAST (blast.ncbi.nlm.nih.gov) with an E-value range of 1×10^{-39} - 2×10^{-41} . To isolate *P. aeruginosa* PAO1H2O from wastewater, a serial dilution of wastewater was plated on Pseudomonas Isolation Agar (PIA) (Fluka Analytical, 172208) and incubated for 24 hours at $37^\circ\text{C} \pm 2^\circ\text{C}$. Positive *P. aeruginosa* colonies were identified by positive fluorescence under black light and the presence of pyoverdine (green) pigmentation. Further

confirmation was performed with 16s RNA PCR amplification (see Table 7.1 for primer selection). To complete 16s RNA PCR amplification, genomic DNA was extracted with MoBio (Carlsbad, CA, USA). Each PCR reaction contained 30 ng genomic DNA, which was measured with the Qubit 3.0 fluorometer (Invitrogen by Life Technologies, New Jersey NY) as well as 12.5 μ L SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA), 200 nM primers (obtained from a primer stock of 10 μ M) and filled to 25 μ L with Sigma water (Sigma-Aldrich, Saint Louis, MO). Cycling parameters included an initial denaturation at 95.0°C for 5 min, followed by 40 cycles of 95.0°C for 10s, 60.0°C for 20s and 72.0°C for 15s. A melt curve was also performed for quality control with increasing temperatures from 65.0°C to 95°C in 0.5°C increments for 5s. All reactions were performed in triplicate and analyzed with the Bio-Rad CFX96-C1000 Real-Time PCR system and the Bio-Rad CFX Manager 3.1. PCR amplicon was cleaned with QIA quick PCR Purification Kit (QIAGEN Sciences Maryland, USA). Representative amplification and melt curve can be seen in Figure 7.2. Gel electrophoresis was run on 2.5% agarose gel at 90 volts for 70 min and stained with 0.004% (w/v) ethidium bromide in 1X TAE for 30 minutes to demonstrate the size and quality of the PCR fragment, see Figure 7.3. High-throughput DNA sequencing was performed with the Applied Biosystems 3730 DNA Analyzer by StemCore Laboratories (Ottawa, ON, Canada). Stock solutions of both strains of *P. aeruginosa* solutions were revived overnight at 37°C $\pm$ 2°C and washed 3X with PBS before use.

Figure 7.2: (a) 16s RNA PCR amplification curve of *P. aeruginosa* isolated from wastewater $C_q = 12.19$ (blue circles) and non-template control $C_q = 24.49$ (red crosses) and (b) associated melt curve of 16s RNA PCR amplification of *P. aeruginosa* isolated from wastewater (blue circles) and non-template control (red crosses)

Figure 7.3: 2.5% agarose gel stained with ethidium bromide Lane 1: 50 bp ladder; Lane 2: intentionally left empty; Lane 3: 16s amplicon from *P. aeruginosa* isolated from wastewater, photo taken with Canon 60D EOS with EF-S 18-55mm f/3.5-3.6 IS lens with Kenko YA3(orange) 50-56, 58mm and a UVIR filter

7.2.5 Culture methods

Membrane filtration procedure outlined in Protocol 9222B of *Standard Methods* was used to enumerate each bacteria type using membrane filtration units by Advantec (Japan) along with 0.45 μm , 47 mm S-Pak™ membrane filter by Millipore water (Billerica, MA, USA) (Eaton et al., 2005). Filtration was vacuum assisted with a Vacuum Pressure Pump by Barnant Co, model number 400-1901 (Barrington, IL, USA). Each dilution was performed with a minimum of three replicates diluted in PBS (Eaton et al., 2005). *E. coli* was enumerated on Bacto™ EC Medium with MUG by BD (Mississauga, ON, Canada) with 1.5% (w/v) Agar Laboratory Grade ARG003.500 by Bishop (Burlington, ON, Canada), *P. aeruginosa* was enumerated on PIA and *B. subtilis* on TSB with added agar.

7.2.6 PMA protocol

A volume of 5 μ L of 20 mM Propidium monoazide (PMA) (Biotium, Hayward, CA, USA) dissolved in 20% dimethyl sulfoxide (DSMO) (99.9%, Caledon Laboratory Chemicals, Georgetown, ON, Canada) was added to 0.95 mL of treated sample, mixed gently with a vortex mixer for 5 seconds and incubated in the dark at room temperature for 10 minutes. PMA-sample mixtures were then exposed to 150-watt halogen bulb for 10 minutes on ice and gently mixed by hand every two minutes.

7.2.7 DNA extraction and quantification, and qPCR protocol

Genomic DNA was extracted using Mo-bio PowerSoil DNA Isolation Kit (Carlsbad, CA, USA), which includes a bead-beating step. Sample volumes of 1 mL were centrifuged for 3 min at 10,000xg to form a pellet and the supernatant discarded. Samples were processed according to the instructions of the isolation kit with the exception that all incubation periods were increased from 5 to 10 minutes. DNA extracts were quantified using the Qubit 3.0 Fluorometer (Life Technologies, Eugene, OR) and stored at -20°C until used for qPCR analysis.

The qPCR protocol minimized false positives by using native Taq polymerase and included magnesium chloride (MgCl₂), DSMO (99.9%, Caledon Laboratory Chemicals, Georgetown, ON, Canada), and Bovine Serum Albumin (BSA) to minimize non-specific amplification due to humic acids in wastewater (Kibbee et al., 2013). The protocol amplified the *uidA* gene (see Table 7.1 for details) of *E. coli*, which encodes for the β -glucuronidase enzyme, that has been shown appear in 97.7 % of *E. coli* isolates from treated and raw water samples (Martins et al., 1993) and appears only once per genome

(Taskin et al., 2011). Reactions were performed with: 5 μ L DNA template, 2.5 μ L 10xPCR Buffer (Invitrogen, Carlsbad, CA, USA), 1.25 μ L 50mM MgCl (Invitrogen, Carlsbad, CA, USA), 0.5 μ L 10 mM dNTPs (Invitrogen, Carlsbad, CA, USA), 1.25 μ L 5% DMSO min (99.9%, Caledon Laboratory Chemicals, Georgetown, ON, Canada), 0.625 μ L of 4 μ M stock of forward primer, 0.625 μ L of 4 μ M stock of reverse primer, 1 μ L 10 μ g/ μ L BSA, 20X EvaGreen (Biotium Inc., Fremont, CA, USA), 0.5 μ L DNA Taq Polymerase (Native, Invitrogen, Carlsbad, CA, USA) and filled to 25 μ L with DNA-free water (Sigma-Aldrich, St. Louis, MO, USA). Cycling parameters were as followed: denaturation at 95.0°C for 2 minutes, followed by 40 cycles of 95.0°C for 10 s, 61.4°C for 20s and 72.0°C for 15s. Subsequent to every qPCR run a melt curve was performed for quality control purposes with increasing temperatures from 65.0°C to 95°C in 0.2°C increments with each increment lasting for 5s to assure that consistent amplification was occurring. A representative amplification (Figure 7.4-a) and melt curve (Figure 7.4-b) of a serial dilution of *E. coli* spiked wastewater used to create a standard curve, see Figure 7.4-c. Melt curve demonstrates a single peak at 84.6°C $\pm$ 0.2°C and no non-specific amplification, as demonstrated by the single peak. All reactions were performed in triplicate and analyzed with the Bio-Rad CFX96-C1000 Real-Time PCR system and the Bio-Rad CFX Manager 3.1. The relative quantification method was used to throughout the study to perform the qPCR analysis. Standard curves were created by serial diluting initial *E. coli*-spiked wastewater experimental matrix in deionized water and included in each qPCR run. Using the relative quantification method, results from qPCR and PMA-

qPCR were converted to calculated colony forming unit equivalents (CCE) to allow for a better comparison of results.

Figure 7.4: Representative (a) *uidA* amplification curve of serial dilution of *E. coli* spiked in wastewater, showing linear range $C_q = 17.10, 20.43, 24.56, 27.78, 31.04$ (blue line) with positive control *E. coli* $C_q = 12.12$ (green Xs) and no template control $C_q = 33.44$ (red circles); (b) associated melt curve of (blue line), positive control (green Xs) and no template control (red circles); (c) resulting standard curve for converting C_q to CCE/mL

Table 7.1: PCR primers used in this study

Genetic Target	Primer	Sequence (5' → 3')	PCR Product Size (bp)	Reference
16s	341	CCTACGGGAGGCAGCAG	193	Muyzer, de Waal, and Uitterlinden, 1993
	534	ATTACCGCGGCTGCTGG		
<i>uidA</i> gene	UAL 1939b	ATGGAATTTGCGCGATTTTGC	187	(Heijnen and Medema, 2006b)
	UAL 2105b	ATTGTTTGCCTCCCTGCTGC		

7.2.8 Live/dead viability stain

LIVE/DEAD BacLight Bacterial Viability Kit L13152 (Molecular Probes, Eugene OR) was used to visualize viable and nonviable cells. Dyes from the kit were hydrated in a common 5 mL of deionized water and stored at 4°C in a sterilized dark glass container for up to one month. To perform the staining, samples were mixed in equivolume amounts with hydrated dye mixture, gently vortexed for 5 seconds and incubated in the dark at room temperature for 10 minutes. Volumes of 18 µL were placed on glass slides with cover slip and visualized with Nikon Eclipse TE (Nikon, Montreal QC) microscope equipped with Intensilight C-HGFI fluorescent illuminator (Nikon, Montreal QC) and GFP-1, Cy3, GFPHQ filters (Nikon, Montreal QC). Images of were taken with the Q-imaging Retiga Exi Fast 1394 camera (Nikon, Montreal QC) and processed with NIS-Elements software (Nikon, Montreal QC). Live or viable cells appeared green and dead or nonviable cells appeared red. For each sample, slides were scanned visually, and a count of the total and live cells was recorded. Three representative fields of view were photographed and further analyzed.

7.2.9 Experimental matrix

Secondary effluent wastewater was collected weekly from a wastewater treatment plant in Ontario, Canada in sterile 4-L Nalgene containers and kept at 4°C until used. The treatment plant has a conventional treatment train serving approximately 883,000 residents with average daily flows of 390,000 m³/day. Wastewater was spiked with *E. coli* culture, which was grown overnight in TSB with added agar at 37°C ± 2°C, at a ratio of 2:100 to achieve consistent starting concentrations of 7 Log CFU/mL. *B. subtilis*

suspensions were spiked into autoclaved (121°C for 15 minutes) secondary wastewater at a ratio of 2:100 to achieve starting concentration of 5 Log CFU/mL.

7.2.10 Minimal media

To prepare minimal media solutions 20 mL 5X M9 salts (Sigma-Aldrich, ST. Louis, MO), 0.2 mL autoclaved 1M $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ (BioShop, Burlington, ON, Canada), 0.01 mL autoclaved 1M calcium chloride (CaCl_2) (Fisher Scientific, Rochester, NY) was added to 75 mL of autoclaved deionized water, and diluted to 96 mL (Sambrook and Russell, 2001). A volume of 9.6 mL was aliquoted into sterilized 15 mL conical centrifuge tubes to which either, no food (negative control) , 0.2 mL filtered-sterilized solution of 20% glucose (positive control) (Sigma-Aldrich, ST. Louis, MO) or 0.2 mL filtered-sterilized 20% acetic acid (Sigma-Aldrich, St. Louis, M) was added. Solutions were spiked with cultures of *E. coli* or *P. aeruginosa* to achieve an initial bacterial concentration of approximately 10^9 CFU/mL. All minimal media solutions were incubated for 24 hours at $37^\circ\text{C} \pm 2^\circ\text{C}$. Bacteria were enumerated using culture method described in Section 6.2.3.

7.3 Results and Discussion

7.3.1 Culturability of *E. coli* after treatment

Figure 7.5 presents the effectiveness of wastewater disinfection with PAA, UV and the combined use of UV and PAA as determined by culturing indicator organism *E. coli*. Figure 7.5-a demonstrates the loss of culturable *E. coli* with increasing concentrations of PAA from 1.2 to 11.6 mg/L and contact times of 15 minutes. Results demonstrate that PAA reached its maximum effectiveness at 2.3 mg/L PAA where culturable *E. coli* were

reduced from 7.0 Log CFU/mL to 1.3 Log CFU/mL. Additional increases in the concentration of PAA did not translate to greater reductions in *E. coli*. To minimize the use of the chemical oxidant PAA, 2.3 mg/L PAA was selected as the test concentration. In certain experiments, a higher concentration of 11.6 mg/L PAA was also included.

Figure 7.5-b compares the single and combined use of UV fluences of 40 and 60 mJ/cm² with 2.3 mg/L PAA. A fluence of 60 mJ/cm² is most often seen as the design fluence at WWTP and the lower fluence of 40 mJ/cm² is often seen in water treatment plants. The latter was included in the study to demonstrate the potential reduction of the UV fluence when supplementing UV with PAA. The first series of columns (Figure 7.5-b) demonstrates that UV fluence of 40 mJ/cm² and 60 mJ/cm² reduced culturable *E. coli* from approximately 7.0 Log to 1.7 and 1.4 Log CFU/mL, respectively. The addition of 2.3 mg/L PAA further reduced the culturability of *E. coli* to below the detection limits, providing an additional 1.7 Log in disinfection capacity.

Commercial solutions of PAA are equilibrium mixtures composed primarily of PAA, acetic acid, HP, and water. It is unavoidable to add both acetic acid and HP to wastewater when adding PAA. Wagner et al., (2002) compared the use of PAA and HP for wastewater disinfection and found that to achieve a 2-Log reduction in fecal coliforms concentrations of HP were ten times that of PAA, indicating that PAA was a much more efficient disinfectant compared to HP. However, when HP is used in combination with UV, there is a potential to create oxidative species that could improve the effectiveness of the disinfection treatment. The purpose of the third series of

columns (in Figure 7.5-b) was to determine if HP, which is added as part of the PAA mixture, was contributing to the effectiveness of the PAA treatment. The experiment was completed by adding an equivalent amount of HP that is found in 2.3 mg/L as PAA of the PAA mixture and combined this amount of HP with UV fluences of 40 and 60 mJ/cm². Results demonstrate that the combined use of UV and HP outperformed the use of UV alone and contributed an additional reduction of 0.5 Logs CFU/mL. Subtracting this value from previous results of the combined use of PAA and UV indicates that 1.2 Log reduction (or approximately 71%) is being contributed by PAA and other compounds of the PAA mixture excluding HP.

Figure 7.5: Inactivation of *E. coli* in secondary wastewater with initial concentrations 6.97 Log CFU/mL after: (a) PAA treatment with 1.2, 2.3, 3.5, 4.6, 11.6 mg/L (b) UV fluences of 40 mJ/cm² and 60 mJ/cm² used alone (first series of columns), in combination with 2.3 mg/L PAA (second series of columns), and in combination with 3.1 mg/L HP (third series of columns)

7.3.2 Culturability of *B. subtilis* after treatment

Compliance testing at WWTPs is typically completed using culture methods that detect enteric indicator organisms, such as fecal coliforms, total coliforms, and *E. coli*. These organisms point to the presence of sewage contamination but do not provide any insight to levels of more resistant bacteria or pathogens that could remain after disinfection. This section presents results from experiments that were carried out using *B. Subtilis*. *B. Subtilis* is not native to wastewater. However, it is a spore-forming bacteria and has been used as a surrogate for oocyst-forming *Cryptosporidium parvum* and spore-forming bacteria *Bacillus anthracis* (Cho et al., 2006a; Larson and Marinas, 2003; Nicholson and Galeano, 2003).

Results show that similar to *E. coli*, UV was effective at reducing concentrations of *B. subtilis* by 3-4 Logs at fluences of 10 mJ/cm² and above, see Figure 7.6-a. These results are also similar to those achieved by Cho et al. (2006) who investigated the effect of UV on *B. subtilis*. Studies that investigated spore-forming intestinal parasites *Encephalitozoon intestinalis*, *Encephalitozoon cuniculi*, and *Enciphalitozoon hellem* observed that depending on the strain that UV fluences between 60 and 190 mJ/cm² of were required to achieve 3.2 Log reduction, indicating that UV may affect spores in a much different way than non-spore-forming *E. coli* (Marshall et al., 2003; Nicholson and Galeano, 2003). The single use of PAA, on the other hand, was not effective on *B. subtilis* at any of the concentrations tested (0.58-11.6 mg/L), see Figure 7.6-b. Botzenheart and Jaax (1985) observed that concentrations as high as 200 mg/L PAA were required to reduce *B. subtilis* by 4-Logs and therefore higher doses of PAA may be required before

an effect is seen. The combined effect of UV and PAA had similar results to results when UV was used alone, see Figure 7.7. The results highlight that while combining UV and PAA would increase the removal of *E. coli*, the combined treatment may not add any additional removal for more resistant bacteria, such as spore-forming bacteria as demonstrated with *B. subtilis*.

Figure 7.6: Inactivation of a mixed population of *B. subtilis* vegetative and spore cells in autoclaved secondary wastewater with initial concentration of 5.74 Log CFU/mL subsequent to (a) UV at UV fluences of 0, 10, 20, 40 and 60 mJ/cm², and PAA concentrations of 1.2, 2.3, 3.5, 4.6, 11.6 mg/L

Figure 7.7: Inactivation of *B. subtilis* in autoclaved secondary wastewater with initial concentration of 5.74 Log CFU/mL subsequent to the use of UV fluence of 40 mJ/cm² and 60 mJ/cm² alone and in combination with 2.3 mg/L PAA

7.3.3 Viability of *E. coli* after treatment

There is evidence indicating that sub-lethal concentrations of chemical oxidants may trigger a VBNC-state in bacteria where a portion of bacteria remain viable but are unable to be cultured on standard media (Noor et al., 2009; Oliver et al., 2005). While researchers have had difficulty assessing how to resuscitate VBNC cells (Barcina and Arana, 2009; Coyle and Ormsbee, 2008; Sachidanandham and Gin, 2009), it has been shown that virulence may be maintained in certain cases (Kana et al., 2008; Oliver et al., 1995a; Sun et al., 2008), and that enteric VBNC can be co-cultured with eukaryotic cells indicating that VBNC cells can potentially play a role in gastrointestinal related illnesses (Senoh et al., 2012).

Results comparing all three methods (culture methods, qPCR, and PMA-qPCR) are shown in Figure 7.8. The first series of columns demonstrates what is typically seen with

culture methods and what was seen in Section 7.3.1. That is that a 5.0-Log reduction in culturable *E. coli* was observed with a PAA concentration of 2.3 mg/L and 5.5-Log reduction observed with 11.6 mg/L as PAA. Supplementing UV with PAA reduced culturable *E. coli* to below detectable limits. Results obtained with the direct use qPCR measures the total quantity DNA extracted from a sample regardless if it originated from a viable cell or not. Results of the qPCR are shown in the second series of columns of Figure 7.8 and representative amplification and melt curve can be seen in Figure 7.9. Results demonstrate that at most a 0.5-Log less reduction in CCE/mL was observed when PAA was used alone. This reduction of DNA may in part be attributed to variations due to the heterogeneous nature of wastewater or in part be attributed to lack of sensitivity of the method. Combined treatments with PAA and UV show reductions of 1 and 1.6 for the combined use of PAA and UV fluence of 40 mJ/cm² and 60 mJ/cm², respectively. UV treatment creates pyrimidine dimers in the cell DNA and which can change the gene sequence (Süß et al., 2009). Treatments that used UV saw a greater decrease in CCE/mL due to the coding of the *uidA* gene that the qPCR was designed to measure and as demonstrated in Chapter 4.

Figure 7.8: Inactivation of *E.coli* in secondary wastewater with initial concentration of 6.97 Log CFU/mL subsequent to treatment of 2.3 mg/L PAA, 11.6 mg/L PAA, the combined treatment of UV fluence of 40 mJ/cm² with 2.3 mg/L PAA, and UV fluence of 60 mJ/cm² with 2.3 mg/L PAA as measured by culture methods, qPCR and PMA-qPCR

Figure 7.9: Representative amplification (a) and melt curve (b) of *E. coli* spiked wastewater without PMA (orange triangles), with PMA (blue squares), positive control (green Xs), and no template control (red circles)

PMA is a dye that intercalates with extracellular DNA as well as DNA from cells with damaged membranes and excludes these two types of DNA from downstream qPCR processing. As such, the PMA-qPCR method measures viable *E. coli*. The detection limit of the PMA-qPCR method was found to be 2.6 Log CCE/mL, which was determined by boiling the initial sample of wastewater spiked with *E. coli* for 10 minutes to achieve

sterility. Sterility was also assured with culture methods (data not shown). While the detection limit may seem high, results were always above the limit, so this did not cause any limitation in detecting the VBNC cells. Results of the PMA-qPCR, which can be seen in the third series of columns of Figure 7.8. Viable *E. coli* after PAA treatments of 2.3 mg/L and 11.6 mg/L was measured to be 4.0 and 4.2 Log CCE/mL, respectively, reduced from 6.9 Logs CCE/mL. Compared to results of the culture methods, there was an additional 2 Log of *E. coli* that was not being accounted for with culture methods at 2.3 mg/L PAA, this difference can be considered to be bacteria in the VBNC-state. When UV and PAA were used in combination, no discernable difference in the reduction of viable *E. coli* was observed when PAA was used with a UV fluence of 40 or 60 mJ/cm². Both treatments reduced viable *E. coli* to between 3.2 and 3.3 Log CCE/mL, respectively.

Concurrently to culturing and PMA-qPCR testing, samples were also treated with the live/dead viability stain. This microbial stain allows for the visualization of live and dead bacterial cells. The test kit contains two molecular stains. SYTO-9 is a green-fluorescent stain that labels the nucleic acid of all bacteria in a sample while the fluorescent stain propidium iodide is only able to penetrate cells with damaged membranes. In theory, green-fluorescing cells would be counted as live or viable and red-fluorescing cells as dead or nonviable. However, in reality, the state of cells was not binary. In many cases after treatment, cells fluoresced with both green and red pigmentation and many appeared yellow or orange. To analyze results, Nikon software was used to extract pixel information of each cell. If a cell contained more green pigmentation, it was counted as live and if it contained more red pigmentation, it was counted as dead. Please see

Appendix 7A for live/dead images. Results of the live/dead viability analysis are shown in Figure 7.10. The first point to note is that the scale is not on a log scale, as is the case with previous methods. The live/dead viability stain allows for visualization of the mixed microbial population in wastewater and is not limited to *E. coli*. Results show a similar trend to what was observed in Figure 7.8. There was a general decrease in the number of live and total number of cells with each treatment. However, a large number of viable cells was observed indicating the proportion of bacteria that remained after treatment.

Figure 7.10: Number of live and dead cells as determined with the live/dead viability stain of secondary wastewater spiked with *E.coli* at initial concentration of 6.97 Log CFU/mL subsequent to treatment of 2.3 mg/L PAA, 11.6 mg/L PAA, the combined treatment of UV fluence of 40 mJ/cm² and 60 mJ/cm² with 2.3 mg/L

7.3.4 Regrowth of *E. coli* after treatment

Regrowth experiments were performed to determine if *E. coli* would regain culturability given time following the individual and combined treatment of UV and PAA. Figure 7.11 demonstrates results from culturing *E. coli* 0, 2, 6, 24 and 48 hours after treatment. The figure shows that up until 6 hours, there is little to no change in the concentration of bacteria and no observable regrowth occurred for any of the treatments. In addition, no change was observed in the concentration of bacteria in the initial wastewater sample. After 24 hours, regrowth was observed in all samples with the exception of initial wastewater and continued until the end of the experimental period of 48 hours. After 24, a decrease of 0.4 Logs was observed in the initial wastewater sample and after 48 hours a total decrease of 1.6 Logs was observed. These results indicate that wastewater itself was not able to sustain *E. coli* growth or that a loss of culturability was observed. It is also interesting to note that the combined use of UV and PAA did not reduce culturable cells to below detection limits as in previous sections. This may be due to variation in the characteristics of wastewater, which was collected weekly.

Figure 7.11: Regrowth of *E. coli* in secondary wastewater after treatment with 2.3 mg/L PAA, 11.6 mg/L PAA, the combined treatment of 40 mJ/cm² UV and 2.3 mg/L PAA and the combined treatment of 60 mJ/cm² UV and 2.3 mg/L PAA. Arrows indicate that actual concentrations are higher than reported.

When treated with PAA, regardless of the use of UV or not, an increase in culturability was observed after 24 hours. Cells that are able to regain culturability may in part be VBNC bacteria. In the literature, observations of regrowth after treatment with PAA seem to depend on the type of bacteria examined and the concentration of PAA. Huang et al. (2013) observed regrowth of heterotrophic, as well as tetracycline- and chloramphenicol-resistant bacteria, at concentrations of 2 and 5 mg/L PAA but not at 10 mg/L and above. Other antibiotic-resistant bacteria were also studied and no regrowth was observed up to 22 hours (Huang et al., 2013). However, regrowth may have been observed if the regrowth period were to be extended. Antonelli et al. (2006) examined the regrowth of coliform and heterotrophic bacteria following wastewater disinfection with PAA at doses of 2-15 mg/L after 5, 24 and 29 hours. The authors observed no

regrowth of coliform bacteria; however, regrowth of heterotrophic bacteria was observed when wastewater was treated with 2 mg/L PAA but not for any of the higher doses and only when measured with culture methods. When regrowth was examined using flow cytometry, no regrowth was observed. It was, however, observed that flow cytometry consistently measured higher concentrations of heterotrophic bacteria than what were measured with culture methods. The authors hypothesized that stress from low-dose disinfection caused a loss of culturability in certain cells, which remained viable and observable by flow cytometry. Results presented Figure 7.11, higher regrowth was observed with lower concentrations of PAA. However, regrowth was still observed when using higher concentrations of PAA of 11.6 mg/L as well as when treatment of PAA and UV were combined.

Lazarova et al. (1998) also examined regrowth and offered additional insights into the cause of regrowth observed after treatment with PAA. Treatments with PAA were shown to increase the total organic carbon and biodegradable fraction of dissolved organic carbon (Lazarova et al., 1998) which could provide a carbon source for surviving bacteria to replicate. In addition, the authors observed that fecal coliforms maintained enzyme activity following treatment with PAA. In fact, enzyme activity was shown to increase after culturable cell concentration decreased. This could support the idea that cells that are no longer cultivable can repair damage and cause regrowth after a lag period.

7.3.5 Contribution of acetic acid to regrowth

The commercial formulation of the PAA used throughout experimentation is an equilibrium mixture of 15% PAA, 23% HP, 16% acetic acid, and water. Acetic acid is introduced with the addition of the PAA and is a decomposition product as well. Acetic acid is a simple hydrocarbon chain and has been suggested by other researchers as a potential food source for bacteria and a contributor to the regrowth observed after disinfection with PAA (Antonelli et al., 2006; Huang et al., 2013). To test this hypothesis, minimal media was supplemented with glucose (positive control), acetic acid, or was not supplemented with a carbon source (negative control). *E. coli* and two strains of *P. aeruginosa* (an ATCC strain and a strain isolated from wastewater) were selected as test organisms. *P. aeruginosa* is prevalent in the environment, is an opportunistic pathogen that has been shown to have the ability to develop antibiotic resistance (Wong and Kassen, 2011).

Results presented in Figure 7.12 show a similar trend between both strains of *P. aeruginosa* and *E. coli*. Without a carbon source, both bacterial strains were able to maintain initial concentrations, but were unable to propagate. Minimal media is an isotonic solution that can sustain bacterial populations, but requires a carbon source to allow for the production of essential amino acids as well as growth. Supplementing with glucose allowed propagation of bacteria as seen by a 1-Log increase in concentrations after 24 hours. If acetic acid was able to act as a carbon source, similar results to those would have been observed with glucose. However, supplementation with acetic acid decreased the concentration of bacteria by approximately 2-Log indicating that acetic

acid negatively affects the growth of cells and is not a contributing factor to the regrowth observed in the previous section. Regrowth observed in the previous section (Figure 7.11) is more likely due to the increases in the organic load as well as changes in the bio-available carbon that occurred during treatment as suggested by Lazarova et al. (1998).

Figure 7.12: *E. coli* and *P. aeruginosa* in minimal media supplemented with no carbon source, 2% glucose or 2% acetic acid

7.4 Conclusion

The purpose of this research was to investigate the use of PAA to supplement UV disinfection. Culture methods showed that UV supplemented with PAA was equally (in the case of *B. subtilis*) or more (in the case of *E. coli*) effective than using either treatment alone for the reduction of culturable bacteria. Coupling disinfection methods,

allows different disinfection mechanisms to be applied, which broadens the disinfection capacity and flexibility of WWTPs. In addition, the potential formation of hydroxyl radicals, from PAA in the presence of UV, can also increase efficiencies.

The PMA-qPCR molecular method highlighted that a large number of bacteria are triggered into a VBNC state following disinfection. The combined treatment increased reduction of viable *E. coli*, however, between 3 and 5 Log CCE/mL remained.

Regrowth was observed after 24 hours following all treatments with PAA regardless if used in combination with UV likely due to the oxidation of organic carbon to more bioavailable sources.

7.6 References

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Appendix 7A: Live dead images and pixel analysis

Figure 7A. 1: Pixel analysis and accompanying image from live/dead viability stain of the initial wastewater sample

Figure 7A. 2: Pixel analysis and accompanying image from live/dead viability stain of the wastewater treated with 2.3 mg/L PAA

Figure 7A. 3: Pixel analysis and accompanying image from live/dead viability stain of the wastewater treated with 11.6 mg/L PAA

Figure 7A. 4: Pixel analysis and accompanying image from live/dead viability stain of the wastewater treated with UV fluence of 40 mJ/cm^2 and 2.3 mg/L PAA

Figure 7A. 5: Pixel analysis and accompanying image from live/dead viability stain of the wastewater treated with UV fluence of $60 \text{ mJ/cm}^2 + 2.3 \text{ mg/L PAA}$

Chapter 8

8 Conclusions and Future Work

The major findings of this study and areas for further study are presented below.

In **Chapter 4** culturable and viable *E. coli* were compared following individual treatment use of sodium hypochlorite (NaOCl) and chlorine-free chemicals HP, PAA, and ferrate(VI) as well as UV at concentrations or fluences that would be either used at a WWTP or that were suggested in the literature for wastewater disinfection applications. Viable *E. coli* was reduced by 1.4 logs with NaOCl, by 1.5 logs with PAA, and 3 logs ferrate(VI), while the culturability of was reduced to between 6.4 and 7 logs for all treatments. NaOCl and PAA have similar ORPs and disinfection mechanisms. That is, both chemicals are more likely to permeate the cell membrane and have an effect on internal cell components. The similarity in disinfection mechanism may also account for the similarity in reduction in viable bacteria. Ferrate(VI) is a stronger oxidant and may have a greater effect on the permeability of the cell membrane and a greater affect at reducing VBNC bacteria. However, higher concentrations of ferrate(VI) were used in the study in comparison to those of NaOCl and PAA. Overall, the study found that a significant proportion of *E. coli* enter a VBNC state following chemical disinfection.

Continuing with the theme of investigating VBNC bacteria, culturable and viable *E. coli* were compared following the use of UV and ferrate(VI) when used alone and in combination (**Chapter 6**) as well as the use of UV and PAA used alone and in combination (**Chapter 7**). The combined use of UV and ferrate(VI) offered an additional

80% reduction in culturable *E. coli* compared to the use of UV alone and a 2-log reduction of viable *E. coli* compared to a 1-log reduction achieved with ferrate(VI) alone. The combined use of UV and PAA reduced viable *E. coli* by 0.6 CCE/mL while culturable *E. coli* were reduced to below detection limits. These studies demonstrated that combining disinfection methods could help to decrease the concentration of VBNC bacteria after treatment.

Future work required to investigate the VBNC state of bacteria following disinfection more fully includes an investigation of higher concentrations of oxidants and as well as a comparison of the effectiveness of chemicals with low ORPs to those with high ORPs. Research has been able to show that VBNC bacteria retain virulence; however, further work is required to determine to what degree virulence is maintained and what risk do VBNC bacteria pose to public health. The VBNC state of different microorganisms after disinfection should also be tested.

The culturability of indicator organisms *E. coli* and coliform bacteria were compared to that of non-traditional spore-forming bacteria *C. perfringens* and *B. Subtilis*. In **Chapter 5** the use of ferrate(VI) in combination with UV further reduced the population of culturable coliform bacteria by 80% when compared to using UV alone without affecting effluent wastewater characteristics. Results of **Chapter 6** demonstrated that the combined use of UV and ferrate(VI) had no additional benefits for the reduction of culturable *C. perfringens* at concentrations tested. In **Chapter 7** the use of UV and PAA for the reduction of culturable *E. coli* and *B. subtilis* was investigated. The combined use

of UV and PAA reduced *E. coli* to below detectable levels but PAA had no effect on the reduction of culturable *B. subtilis* nor did the combined use of PAA and UV when compared to the use of UV alone. Overall, the studies showed that there were additional gains when combining UV with ferrate(VI) or PAA for the reduction of culturable indicator organism but no gains were observed in reductions of the spore-forming bacteria studied.

Future work to further investigate non-traditional indicator organisms could vary the strengths of UV and the chemical pair to find a combination that may be more effective.

The VBNC state is a low metabolic state that has been shown to be triggered by a stressor such as low nutrient, oxygen, or temperature conditions, as well as oxidative stress. The third theme explored in this thesis was the changes to culturability that were monitored for 48 hours after treatment. In **Chapter 6** the differences between regrowth, regeneration, and resuscitation were discussed and used to analyze results of monitoring culturability of indigenous coliform bacteria following treatment with UV and ferrate(VI) when used alone and in combination. Results demonstrated that regrowth and resuscitation were observed the soonest after treatment with NaOCl suggesting that low strength oxidants allow for greater risk of regrowth following treatment. Regrowth and reactivation were observed with UV, as expected, and no significant increases in culturable coliform bacteria were found with ferrate(VI). The latter result suggests that stronger oxidants that have a greater effect on the cell wall may prevent the reactivation of damaged cells and resuscitation of VBNC cells. The

combined treatment showed a trend that was similar to that observed with UV indicating that reactivation may contribute to increases in culturability after combined treatment. In **Chapter 7** changes to the culturability of *E. coli* after disinfection with PAA and UV when used alone and in combination were studied. Increases in culturable *E. coli* were observed after a lag period between 6 and 24 hours without additional nutrient supplementation. However, the contribution of the PAA-oxidized organic matter remains to be confirmed as a contributing microbiological regrowth.