Chlorine Dioxide and Ozone Facilitated Disinfection of Selected Bacteria in Aqueous Systems

by

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As the candidate's supervisor, I hereby approve this thesis for submission.

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ABSTRACT

Chlorination is the most commonly used disinfection technology for the control of pathogenic microorganisms in drinking water or wastewater treatment. However, the reactions of chlorine with natural organic matter in water have been found to produce harmful by-products including trihalomethanes, haloacetic acids, and haloacetonitriles. Regulations on these deleterious disinfection by-products keep increasing and have consequently focussed considerable attention on the use of alternative chemical disinfectants. The design and operation of an efficient disinfection system at a water treatment facility aim at providing adequate control of microbial threats and simultaneously satisfying regulatory requirements on by-products. Achieving these require an in-depth understanding of the inactivation kinetics of the disinfectant on potential target organisms.

In this study, the microbial inactivation kinetics of chlorine dioxide (ClO_2) as an alternative chemical disinfectant for water treatment was monitored on two Gram-negative bacterial species: *Escherichia coli* (ATCC 35218) and *Pseudomonas aeruginosa* (ATCC 27853) and a gram-positive: *Staphylococcus aureus* (ATCC29313) under varied conditions of disinfectant concentration, pH, temperature and bacterial density in an oxidant demand free water. Further studies were conducted to investigate the effect of ClO_2 on bacterial outer cell membrane permeability, the cytoplasmic membrane integrity, inhibition of intracellular enzyme activity and changes in cell morphology by TEM to elucidate the bactericidal mechanism of action of ClO_2. In addition, autochnous bacteria from urban wastewater were exposed to chlorine dioxide and the susceptibilities monitored and compared by a culture-dependent heterotrophic plate count technique and culture-independent 16S rRNA gene-directed polymerase chain reaction (PCR) based denaturing gradient gel electrophoresis (DGGE). Furthermore, the influence of four organic solvents commonly discharged from industrial lines into wastewater systems, namely, ethanol, methanol, ethyl acetate and dimethyl sulfoxide (DMSO) on ozone absorption, stability and consequent inactivation of *Escherichia coli* (ATCC 25218) and *Staphylococcus aureus* (29213) in water were also examined.

Chlorine dioxide showed strong and rapid disinfection capabilities at relatively lower dosages with significant influences by pH and temperature. However, the efficiency generally appeared unaffected by changes in bacterial density. The PCR- DGGE technique showed that 1.0 mg/L was sufficient to inactivate three predominant bacterial species from an urban wastewater,

identified as *Arcobacter suis F41*, *Pseudomonas sp strain QBA5* and *Pseudomonas sp B-AS-44*, whereas a significant population of other species such as *Pseudomonas sp CCI2E* was observed to presumably remain viable to 5.0 mg/L chlorine dioxide whilst the heterotrophic plate count method indicated complete elimination of bacteria at 3.0 mg/L. ClO_2 was not found to inactivate bacteria by inflicting gross morphological damages to the cell wall, but instead, increases the permeability of the outer cell membrane, disrupts the integrity of the inner cytoplasmic membrane which leads to the efflux of intracellular contents of the cell and hence, resulting in the overall cell death. The presence of ethyl acetate and DMSO were observed to significantly enhance ozone absorption and stability in water with a consequent increase in bacteria inactivation efficiency whilst methanol-containing water rather accelerated the decomposition of ozone. The findings herein provide further knowledge to enhance the disinfection operations at a water treatment plant when ClO_2 or O_3 is applied

KEYWORDS: Chlorine dioxide; Ozone; Kinetics; Inactivation; *Escherichia coli; Pseudomonas aeruginosa; Staphylococcus aureus*

PREFACE

The experimental work described in this thesis was carried out in the School of Chemistry and Physics, and the Discipline of Microbiology, of the University of KwaZulu-Natal from September 2014 to October 2017 under the supervision of Professors Sreekantha Babu Jonnalagadda and Johnson Lin.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, it is duly acknowledged in the text.

DECLARATION 1 - PLAGIARISM

I, **Isaac Ofori,** declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
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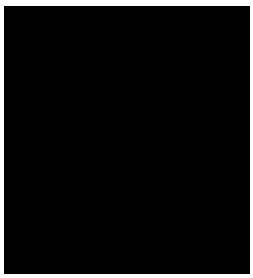
Signed……  Date……

DECLARATION 2- PUBLICATIONS

Details of contributions to publications that form part and/or include research presented in this thesis

Manuscripts published

1. **Isaac Ofori**, Suresh Maddila, Johnson Lin and Sreekantha B. Jonnalagadda (2017) Chlorine dioxide oxidation of *Escherichia coli* in water – A study of the disinfection kinetics and mechanism. *Journal of Environmental Science and Health, Part A* 52(7):598-606. DOI: 10.1080/10934529.2017.1293993
   (Contribution: *Isaac Ofori* designed the study protocol, carried out the experiments, interpreted the results and wrote the initial manuscript draft. Prof S.B. Jonnalagadda and Prof Johnson Lin closely supervised all stages of the work. Dr. S. Maddila is a postdoctoral research fellow working under the supervision of Prof S.B. Jonnalagadda and assisted in compiling and editing the draft of the manuscript.
2. **Isaac Ofori**, Suresh Maddila, Johnson Lin and Sreekantha B. Jonnalagadda (2018). Ozone Initiated Inactivation of *Escherichia coli* and *Staphylococcus aureus* in water: Influence of Selected Organic Solvents Prevalent in Wastewaters**.**
   Chemosphere, 2018. DOI.org/10.1016/j.chemosphere.2018.04.164
   (Contribution: *Isaac Ofori* designed the study protocol, carried out the experiments, interpreted the results and wrote the initial manuscript draft, Prof S.B. Jonnalagadda, and Prof Johnson Lin closely supervised all stages of the work. Dr. S. Maddila is a postdoctoral research fellow working under the supervision of Prof S.B. Jonnalagadda and assisted in compiling and editing the draft of the manuscript.

Manuscripts submitted for review

3. Isaac Ofori, Suresh Maddila, Johnson Lin and Sreekantha B. Jonnalagadda (2017). Inactivation Kinetics and Mechanism of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in water: The case of Chlorine Dioxide:

(Contribution: *Isaac Ofori* designed the study protocol, carried out the experiments, interpreted the results and wrote the initial manuscript draft. Prof S.B. Jonnalagadda, and Prof Johnson Lin closely supervised all stages of the work. Dr. S. Maddila is a postdoctoral research fellow working under the supervision of Prof S.B. Jonnalagadda and assisted in compiling and editing the draft of the manuscript.

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(Contribution: *Isaac Ofori* designed the study protocol, carried out the experiments, interpreted the results and wrote the initial manuscript draft, Prof S.B. Jonnalagadda, and Prof Johnson Lin closely supervised all stages of the work. Dr. S. Maddila is a postdoctoral research fellow working under the supervision of Prof S.B. Jonnalagadda and assisted in compiling and editing the draft of the manuscript

LIST OF CONFERENCE PRESENTATIONS

- **Isaac Ofori**, Johnson Lin, and Sreekantha B. Jonnalagadda. Kinetics and Mechanism of Chlorine Dioxide Disinfection of *Escherichia coli* in aqueous systems. ***Gordon Research Conference: Bacterial Cell Surfaces,* June 26 – July 1, 2016, Mount Snow in West Dover, VT, United States.** *Poster presentation*

- **Isaac Ofori**, Johnson Lin, and Sreekantha B. Jonnalagadda. The Kinetics and Mechanism of Chlorine Dioxide Disinfection of *Escherichia coli* and *Pseudomonas aeruginosa* in Aquatic Systems. ***University of KwaZulu-Natal College of Agriculture, Engineering and Science Postgraduate Research Symposium***, **29th November 2016**, Howard College, Durban. *Oral presentation*

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To my grandmother, parents, and siblings, I appreciate all the support and sacrifices you had to go through to get me this far.

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DEDICATION

This work is dedicated to

my grandmother *Madam Monica Kyeraa and* my lovely wife *Mrs. Harriet Ofori*

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LIST OF ABBREVIATIONS

ARB	Antibiotic resistant bacteria
ARG	Antibiotic resistant genes
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
COD	Chemical oxygen demand
CPR	Chlorophenol red
DAEC	Diffusely Adherent *E. coli*
DBP	Disinfection by-products
D/DBP	Disinfection and Disinfectants By-products Rule
DGGE	Denaturing gradient gel electrophoresis
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPD	*N*, *N*-diethyl-*p*-phenylenediamine
DOM	Dissolved Organic Materials
EAEC	Enteroaggregative *E. coli*
EDTA	Ethylenediaminetetraacetic acid
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ESS	Error Sum of Squares
ETEC	Enterotoxigenic *E. coli*
FAS	Ferrous Ammonium Sulphate
HAA	Haloacetic acids (HAA),

HAN	Haloacetonitriles
HEPES	N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid
HPC	Heterotrophic plate count
IOD	Instantaneous ozone demand
LB	Lysogeny broth
LGB	Lissamine Green B
LPHO	Low pressure-high-output
LPS	Lipopolysaccharides
MCL	Maximum contaminant level
NADH	Nicotinamide adenine dinucleotide
NFWTP	Niagara Falls Water Treatment Plant
NOM	Natural organic materials
ODF	Oxidant demand free
OM	Outer membrane
ONP	*o*-nitrophenol (ONP)
ONPG	*o* -nitrophenyl-β-D-galactopyranoside
OUT	Operational taxonomic unit
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMA	Propidium monoazide
PPCP	Pharmaceuticals and personal care products
RNA	Ribonucleic acid
rRNA	ribosomal Ribonucleic acid
SS	Suspended solids

SWTR	Surface Water Treatment Rule
TEM	Transmission Electron Microscopy
THM	Trihalomethanes (THM),
USEPA	United States Environmental Protection Agency
VBNC	Viable but non-culturable
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

1.1 Background

Access to potable water and clean sanitation is considered, an essential human right for the enjoyment of life[1]. Recent global reports have shown significant improvements in access to safely managed drinking water. Approximately, 71% (5.2 billion) of the global population are estimated to have access to potable water[2]. However, it is also estimated that over 660 million people still rely on unimproved water sources containing various forms of deleterious contaminants for drinking purposes[2]. Various sources of potable water include; rivers, lakes, streams, aquifers and seawater. Besides these, the treatment of municipal and industrial wastewaters has become an attractive alternative, for the provision of potable water and the supply of water for agriculture purposes in the face of increasing populations, industrialisation and adverse effects of climate change.

Waterborne diseases persist as critical global public health burden and are estimated to account for over 2.2 million deaths annually, predominantly in children under 5 years in developing countries[3]. The economic impacts of waterborne outbreaks including, but not limited to health care cost, response approaches and loss of productive hours are also quite considerable. For instance, the outbreak of *Cryptosporidium hominis* infection in Ireland in 2007 was estimated to cost about $ 22.44 million[4]. The famous Walkerton water crisis in Canada in 2000, which involved an outbreak of pathogenic *Escherichia coli* O157: H7 from a municipal water was considered to have cost nearly $155 million[5].

These diseases are usually caused by contaminants in water including organic and inorganic chemical species, as well as microbial or pathogenic organisms. However, on the basis of the high evident water-related health problems linked to the ingestion of microbial or biological contaminants, the World Health Organization (WHO) considers the hazards posed by pathogenic agents as a primary health concern in both developed and developing countries[1]. Other transmission routes involve person-to-person contacts, food intake, water drops, aerosols and exposure by bathing.

The major groups of waterborne pathogens are bacteria, viruses and parasites (protozoa and helminths). Common bacterial species of considerable concern in water include; *Campylobacter jejuni, Escherichia coli, Legionella spp, Pseudomonas aeruginosa, Salmonella typhi, Shigella, Staphylococcus aureus*, and *Vibrio cholerae*. Waterborne viruses such as Adenovirus, Hepatitis A and E viruses, Norovirus, Enterovirus, Rotavirus, and Sapovirus have also attracted significant attention. The protozoa spp of interest in water are *Cryptosporidium*, *Giardia*, *Entamoeba histolytica*, *Cyclospora cayetanensis* and *Naegleria fowleri*. These organisms are responsible for a range of diseases such as cholera, typhoid fever, amoebic dysentery, gastroenteritis, giardiasis, legionellosis, cryptosporidiosis and various forms of viral infections[1]. Particularly, in South Africa, series of reported outbreaks of legionellosis in recent times have attained considerable attention both locally and internationally and of utmost concern to public health[6].

Due to the potential adverse consequences of waterborne outbreaks on a population, the regulatory requirements for their removal in drinking water are quite high. For instance, according to the Surface Water Treatment Rule (SWTR), the maximum contaminant level goals set for *Legionella*, *Giardia lamblia*, *Cryptosporidium*, virus and total coliform is zero[7]. To meet such requirements, multi-barrier strategies which include the protection of the source water, selection and operation of suitable treatment processes and management of the water through the distribution systems are highly recommended. However, the disinfection stage is pivotal among all the treatment processes aimed at ensuring microbial safety in either drinking water or wastewater.

Disinfection is the deliberate reduction of the number of viable pathogenic organisms in a system to prevent the transmission of infections. For any potable water treatment process, it is usually the last line of defence for eliminating pathogens before pumping the water into the distribution system[8]. Currently, several methods including physical and chemical technologies are employed to achieve adequate disinfection of water. Common physical disinfection methods include but not limited to thermal treatment, electromagnetic radiations such as ultraviolet radiations, x- rays and γ- radiations, ultrasound technology, filtration through filters capable of retaining microorganisms, and reverse osmosis[1, 9]. The chemical-based methods mainly rely on strong oxidants such as chlorine-based disinfectants (Cl_2, HOCl, and OCl^-)

which are collectively termed as free available chlorine or chlorination. Other chemicals include chloramines, chlorine dioxide, ozone, hydrogen peroxide and peracetic acids.

Among these disinfection methods, chlorination is the most widely used technology worldwide[1]. Chlorination has a long history in water application, beginning from the early years of the 20th century when it was applied in a continuous process as a disinfectant in Middlekerke, Belgium[10]. In fact, public health practitioners suggest that chlorine disinfection of water has been one of the greatest public health care measures in the last century[10]. Chlorine is an effective biocidal agent against a wide range of microbial targets such as bacteria, viruses and protozoa. As a bactericide, it is capable of oxidising cell membranes, enzymes and DNA of bacteria and efficient at eliminating slime bacteria, moulds and algae in supply reservoirs as well as the mains and walls of storage tanks[11]. Viruses are also readily inactivated by free chlorine. It is more reliable due to its proven track record over a long period of time, safe and easy to handle, cost-effective, relatively more stable and able to leave residuals in the distribution system. Besides the primary role of disinfection, the strong oxidising properties of chlorine also serve other useful purposes such as taste and odour control, removal of Fe^{2+} and $Mn^{2+,}$ bleaching agent of certain organic dyes and prevention of algal growth.

However, the reactions of chlorine with natural organic materials (NOM) in water have been implicated to produce deleterious disinfection by-products (DBP) including trihalomethanes (THM), haloacetic acids (HAA), haloacetonitriles (HAN), haloketones and chlorinated furanones[12-13]. Subsequent epidemiological and toxicological studies have linked some DBP to bladder cancer and adverse reproductive and developmental effects including early term miscarriages and stillbirths among pregnant women[14-15]. Swimmers in pools disinfected by chlorine are also exposed to DBP through dermal absorption or inhalation of volatile THM like chloroform which has been associated with increased risk of asthma among young children[16]. Furthermore, there are other microbial agents like *Cryptosporidium* and *Giardia* which have also been reported to be resistant to conventional chlorination at the concentrations applied in drinking water treatment[17-18].

Due to these concerns and others, regulations on chlorinated DBP keep increasing[19] and hence, efforts at obtaining adequate microbial disinfection and simultaneously meeting regulatory requirements are considerably high. The use of alternative disinfectants in controlling microbial targets in potable water, wastewater and swimming pools whilst minimising the exposure to unwanted DBP has therefore gained substantial attention.

Chlorine dioxide (ClO_2) is considered as a suitable alternative to chlorination for water disinfection. It is effective against bacteria, viruses and protozoa. Unlike chlorine, it does not form the carcinogenic DBP, even though chlorite (ClO_2^-) and chlorate (ClO_3^-) generated as by-products are also regulated moieties in drinking water[20]. ClO_2 is an excellent oxidant for the control of taste and odour producing phenolic compounds and for the removal of iron and manganese in water.

Ozone is another disinfectant that has attracted significant attention and utilised extensively. It is one of the strongest chemical oxidants for disinfecting both drinking water and wastewater. It has been considered as the most effective disinfectant for the inactivation of *Cryptosporidium* and *Giardia lamblia* which are resistant to chlorination[10].

Despite the potency of these available chemical disinfection technologies, there are still growing concerns with their effectiveness at controlling emerging global threats of antibiotic-resistant bacteria (ARB) and antibiotic resistant genes (ARG) in urban wastewaters and even in some cases tap water. In recent times, it is quite becoming established that ARB and ARG are prevalent in drinking and wastewater sources and could inadvertently become routes of potential transmission into human populations[21-23].

It has been suggested that inadequate disinfection owing to the exposure to sub-inhibitory concentrations of chemical disinfection technologies including chlorination and UV could trigger biochemical stress responses, which might facilitate the development and transfer of clinically relevant ARB and ARG[24-28]. That notwithstanding, regulations on the use of chemical disinfectants aimed at minimising the generation of DBP keep increasing. Efforts at enhancing

microbial safety of treated water and simultaneously meeting regulatory requirements of the applied disinfectants, require an appropriate design and optimization of the operational conditions based on reliable disinfection kinetics data of the disinfectant.

Presently, extensive data characterising the disinfection kinetics of chlorine dioxide on bacteria under the influence of varied operational conditions such as disinfectant concentration, pH, water temperature, microbial density and organic matter content is limited. Such data form the bases of the design and operations of an effective disinfection regime at a water treatment facility and to minimise the suitable conditions of resistance. Moreover, unlike chlorine and ozone, research data on the primary bactericidal mechanism of chlorine dioxide is sketchy and quite contradictory.

In view of the increasing reports of resistant bacteria in source waters, an understanding of the fundamental mechanism of action of chlorine dioxide as an alternative water disinfectant becomes significant. It provides insights into the basis of the activity which could eventually enhance the design and synthesis of novel chemical disinfectants and the prediction of possible development of microbial resistance. In addition, it also provides the bases for predicting a potential synergy or otherwise of a disinfection process when chemical disinfectants are applied in combination[29]. Furthermore, there are also limitations with the routinely employed culture-based techniques of monitoring disinfection efficiency and prospecting for potential resistant strains of bacteria in a complex system like urban wastewater[30]. Relevant research on alternative techniques in the context of the wider global efforts of monitoring ARB and ARG from various sources is critical.

There are also key research gaps regarding the effect of various water contaminants on ozone absorption and stability in a wastewater system which are critical parameters of its efficiency for the control of microbial agents. For instance, various forms of organic solvents are discharged from industrial processes into wastewater systems, but information on their influences on ozone disinfection efficiency and the oxidation of contaminants is scarce.

1.2 Objectives of the study

Based on the above, this work was conducted on the following objectives:

- Determine the inactivation kinetics of chlorine dioxide on Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* and Gram-positive bacteria *Staphylococcus aureus* under varied conditions of disinfectant concentration, pH, temperature and bacterial density in an oxidant demand free (ODF) water.
- Elucidate the bactericidal mechanism of action of chlorine dioxide on *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*.
- Compare a culture dependent heterotrophic plate count method and a culture-independent polymerase chain reaction (PCR) based denaturing gradient gel electrophoresis (PCR-DGGE) technique to investigate the susceptibilities of autochnous bacteria population from an urban wastewater exposed to varying concentrations of chlorine dioxide.
- Determine the influence of the presence of methanol, ethanol, dimethyl sulfoxide (DMSO) and ethyl acetate as model organic solvents commonly found in wastewater matrices on ozone absorption and stability in water and their consequent effect on the disinfection of *Escherichia coli* and *Staphylococcus aureus.*

1.3 Structure of the thesis

This thesis is written as a series of discrete or standalone manuscripts organized into chapters. Apart from this chapter (1), there are six more chapters.

Chapter 2 deals with a review of relevant literature which entails an overview of common alternative disinfection technologies, fundamental theories of disinfection in water, predominant models of disinfection kinetics, and brief description of the structure of a typical vegetative Gram-positive and negative bacterial cell.

Chapter 3 describes the kinetics of chlorine dioxide inactivation of *Escherichia coli* under varied conditions of oxidant concentration, pH, temperature and bacterial density in an oxidant demand free (ODF) water. It also highlights the bactericidal mechanism of action of chlorine dioxide on *E. coli*.

Chapter 4 shows a further description of the kinetics and mechanism of chlorine dioxide inactivation of bacteria on *Pseudomonas aeruginosa* and *Staphylococcus aureus* in ODF water.

In Chapter 5, a culture-based heterotrophic plate count and a culture-independent PCR based DGGE techniques were compared to assess the susceptibilities of autochnous bacteria population from urban wastewater samples exposed to different concentrations of chlorine dioxide with the view of simultaneously monitoring the efficiency of chlorine dioxide in wastewater treatment and prospecting for potential resistant bacteria strains.

In Chapter 6, the influence of four water-soluble organic solvents commonly discharged from industrial lines into wastewater systems viz; ethanol, methanol, ethyl acetate and dimethyl sulfoxide (DMSO) on the ozone facilitated inactivation of *Escherichia coli* and *Staphylococcus aureus* in water was explored. In addition, the relative stability and absorption of ozone in water containing these organic solvents were also assessed.

Chapter 7 summarises the key findings of this thesis and provides further insight into research gaps and future perspectives.

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CHAPTER 2

LITERATURE REVIEW

2.1 Overview of common alternative water disinfectants

The overarching goal of water disinfection is to eliminate or reduce the threats of potentially harmful pathogenic organisms and prevent the spread of waterborne diseases. This, however, is different from sterilization where complete elimination or killing of all life forms is targeted. Most large-scale treatment plants, especially those employed for public water supplies utilise chemical disinfection methods to achieve adequate disinfection[1].

An ideal chemical disinfectant should possess a broad-spectrum activity against a wide range of pathogenic bacteria, viruses and protozoa. It should also be non-toxic to humans and other animals at the concentrations required and persist or leave residuals in the distribution system to prevent post-treatment regrowth or re-contamination of bacteria during distribution. In addition, it should also be cost-effective, easy to use and to determine its concentration[2]. There should also be an available technology to enhance its adoption for large-scale applications. Due to these desired characteristics, the number of suitable disinfectants for potable water treatment are quite limited, considering the vast number of chemicals that have the potential to kill microbial agents. An overview of some alternative disinfection technologies to conventional chlorination which have gained wider applications in water treatment processes are considered in this section.

2.1.1 Chloramines

Chloramines are class of oxidants, formed when free chlorine (HOCl or OCl^-) reacts with ammonia in a solution. The ammonia is usually applied as either anhydrous ammonia, a liquified gas or aqueous ammonia (NH_4OH); or as a salt $(NH_4)_2SO_4$ or NH_4Cl). Three main species of chloramines (monochloramines, dichloramines, and trichloramines) which are also referred to as combined chlorine are mostly produced[3].

$$HOCl + NH_3 \leftrightarrow NH_2Cl(\text{monochloramine}) + H_2O \quad (1)$$

$$HOCl + NH_2Cl \leftrightarrow NHCl_2(\text{dichloramine}) + H_2O \quad (2)$$

$$HOCl + NHCl_2 \leftrightarrow NCl_3(\text{trichloramine}) + H_2O \quad (3)$$

However, for water disinfection purposes monochloramine is the most desired product because it is more stable, produces less DBP and generates little or no taste and odour problems whilst the other species are volatile and may impart objectionable chlorinous taste and odour to the treated water[2]. The formation of monochloramine during chloramination is largely, a function of the pH and the relative proportions of the reactants. At the pH range, 7.5 – 9, the production of monochloramine is highly favored, but optimal yields are obtained at pH 8.4. Relative concentrations of Cl_2: N ratio of 5:1 by weight is normally employed in practice to optimize the yield of monochloramine[4].

Monochloramine is more stable than free chlorine and is mostly applied as a secondary oxidant to provide more persistent disinfectant residuals in the distribution network to prevent microbial regrowth and post-treatment contamination. They can effectively control bacteria and deeply penetrate and remove biofilms which develop and attach to the inner surfaces of pipes in the distribution system[5]. It has also been found to significantly reduce the formation of regulated DBP,s such as trihalomethanes and haloacetic acids when compared to chlorine[6-7] and this has led to increasing number of public water treatment plants switching to monochloramine.

That notwithstanding, chloramines are in general less effective disinfectants. They are even much weaker against enteric viruses and protozoa including *Cryptosporidium* and *Giardia*[4]. Consequently, they are not applied solely as primary disinfectants in practice but combined with a much stronger disinfectant such as chlorine or ozone. There are also concerns about the effect of chloramines on kidney dialysis patients because they are not easily removed by the membranes of reverse osmosis (RO) in dialysis machines thus resulting in direct oxidative damage to red blood cells and consequent methemoglobin[8]. When discharged into water bodies, chloramine residuals even at low concentrations may be toxic to fish and other aquatic organisms, as well as eutrophication from high nitrogen levels.

2.1.2 Chlorine dioxide

Chlorine dioxide was first produced by Sir Humphrey Davy in 1814 when he reacted sulfuric acid (H_2SO_4) with Potassium chlorate ($KClO_3$). Subsequently, other scientists found several similarities with the properties of chlorine. It is relatively small, volatile but highly energetic gaseous molecule that exists as a free radical monomer even in aqueous solution[4]. It is an oxidant and usually reacts by a single electron transfer mechanism where it gets reduced to

chlorite ion (ClO_2^-). The single electron abstraction from the electron-rich reactive centers of its substrates or organic molecules uniquely makes it a highly selective oxidising agent[9] .

$$ClO_{2(aq)} + e^- \rightarrow ClO_2^- \qquad E^o = +0.954V \qquad (4)$$

The Cl atom in the molecule exists in the +4-oxidation state and therefore vacant outer orbitals have the capacity to accept a total of $5e^-$ to be reduced to Cl^- as shown in the equation below.

$$ClO_2 + 4H^+ + 5e^- \rightarrow 2H_2O + Cl^- \qquad (5)$$

Consequently, the oxidising power of ClO_2 has been theoretically estimated to be ~ 2.63 times that of chlorine, however, in practice this rarely occurs[2]. This is particularly so because the ClO_2^- that is produced is less reactive and hence further reactions requiring the acceptance of $4e^-$ to completely reduce to Cl^- rarely occur.

$$ClO_2 + 2H_2O + 4e^- \rightarrow Cl^- + 4OH^- \qquad (6)$$

Unlike chlorine, chlorine dioxide does not undergo substitution or addition reactions but rather reacts by electrophilic abstraction of an electron.

2.1.2.1 Physical Properties of ClO_2

Chlorine dioxide is a green-yellowish gas at room temperature with a distinct chlorine-like smell and highly soluble in water[10]. The solubility in water is limited to 70 g/L at 20 ºC and at atmospheric pressure[2], but such high concentrations are extremely difficult to achieve. It is estimated to be approximately 10 times more soluble than chlorine in water,[4] however, unlike chlorine it does not hydrolyze but appreciably remains as a gaseous molecule[11]. Meanwhile, due to its highly volatile nature, a vigorous agitation or aeration of its solution can substantially expunge it from the water. Chlorine dioxide boils at 11 ºC and has a melting point of -59 ºC. Its absorbance maximum is at 360 nm.

Aqueous solutions are also sensitive to rapid photolytic decomposition when exposed to sunlight or UV-light to form Cl^-, O_2, ClO_3^-, and other intermediate oxychlorine compounds[9]. It has even been estimated that up to 70 % of the applied dose in a water disinfection basin can be lost within a few seconds if exposed to bright sunlight[12]. Nevertheless, if it is properly stored in the dark and under refrigerated conditions, its strength can be retained for a period running into several months[13].

2.1.2.2 Generation of ClO_2

Chlorine dioxide gas is unstable thermodynamically. Concentrations exceeding 9.5 % in the air could be explosive [14]. However, in aqueous solutions, concentrations below 10 g/L can safely be generated and stored at low temperatures in the dark. When compressed at temperatures exceeding 40 ºC, chlorine dioxide could be extremely explosive and consequently, it is usually not shipped or stored in commercial quantities but rather generated at the point of use[4].

Several generation methods and commercial technologies are employed to produce chlorine dioxide at the site for water disinfection or at the pulp industry for bleaching. For any of these technologies, the distinguishing factor is the chemical feedstock used to generate the ClO_2 gas. An overview of some of the most common generation methods are discussed below

2.1.2.2.1 Acidification of Sodium chlorite

The commonest feedstock chemical for chlorine dioxide generation is sodium chlorite ($NaClO_2$)[15]. This has further been enhanced by the advances in the production of thermally stable solid $NaClO_2$. Sodium chlorite solution can be acidified with either H_2SO_4 at various stoichiometric ratios or with HCl as shown in the reaction equations below:

$$4NaClO_2 + 2H_2SO_4 \rightarrow 2Na_2SO_4 + 2ClO_2 + HCl + HClO_3 + H_2O \quad (7)$$

$$10NaClO_2 + 5H_2SO_4 \rightarrow 8ClO_2 + 5Na_2SO_4 + 2HCl + 4H_2O \quad (8).$$

$$5NaClO_2 + 4HCl \rightarrow 4ClO_2 + 5NaCl + 2H_2O \quad (9)$$

The H_2SO_4 route is about 50 % efficient in terms of the yield whilst HCl could generate about 77 % of the expected stoichiometric yield. HCl is, therefore, most preferred for the acidification process[2].

2.1.2.2.2 Chlorine solution and Chlorite solution

A solution of ClO_2^- reacts with an aqueous solution of Cl_2 or hypochlorous acid (HOCl) to produce ClO_2[4]. This approach usually requires an excess of the Cl_2 solution to lower the pH to ~ 2.8 – 4 to optimize the conversion of the more expensive $NaClO_2$ precursor and more so, to rapidly push the reaction towards completion. When well optimized, an efficiency of 80 – 93 % [4] could be obtained. The low pH effluent, however, could be corrosive.

$$Cl_{2(g)} + H_2O_{(l)} \rightarrow HOCl + HCl \quad (10)$$

$$2ClO_2^-{}_{(aq)} + HOCl + H^+{}_{(aq)} \rightarrow 2ClO_{2(g)} + Cl^- + H_2O \quad (11)$$

2.1.2.2.3 Chlorine gas – Chlorite solution

In this process, the $NaClO_2$ solution (optimally 25 %) reacts with $Cl_{2(g)}$ to rapidly generate near neutral solutions of ClO_2[2]. This method proceeds at a relatively faster rate than the chlorine solution — chlorite solution route. Moreover, it can generate ClO_2 with a purity exceeding 95 %.

$$Cl_{2(g)} + 2ClO_2^-{}_{(aq)} \rightarrow 2ClO_{2(g)} + 2Cl^-{}_{(aq)} \quad (12)$$

2.1.2.2.4 Chlorine gas – Solid sodium chlorite

This involves a reaction of specially processed solid $NaClO_2$ with $Cl_{2(g)}$ under dilute humidity in a sealed reactor[4]. It rapidly produces a highly purified $ClO_{2(g)}$ free from the impurities of $Cl_{2(g)}$, ClO_2^- or ClO_3^-. If the process is well optimized, this method could generate up to 99 % yield efficiency.

$$2NaClO_{2(s)} + Cl_2(g) \rightarrow 2ClO_{2(g)} + 2NaCl_{(s)} \quad (13)$$

2.1.2.2.5 Chlorate reduction by peroxide and sulfuric acid

Sodium chlorate ($NaClO_3$) is reduced by a mixture of reducing agents including concentrated hydrogen peroxide (H_2O_2) and sulfuric acid (H_2SO_4) to produce ClO_2[2].

$$NaClO_3 + H_2O_2 + H_2SO_4 \rightarrow 2ClO_2 + NaHSO_4 + 2H_2O + H_2O \quad (14)$$

Optimised conditions of this method involve an excess of the acid and peroxide to generate a highly acidic effluent and perchlorate ions. This technology has long been applied to produce ClO_2 on a large scale for the pulp bleaching industry; however, it has also been scaled down as well for the water treatment industry.

2.1.2.2.6 Electrochemical-based method

This process comprises the electrolysis of $NaClO_2$ to produce ClO_2[16].

$$ClO_2^- \rightarrow 2ClO_2 + e^- \quad (15)$$

The method has the advantage of making use of only one chemical. However, limited amount of ClO_2 is produced hence, it is usually employed in small-scale installations. Relatively it is more expensive than the other generation methods

2.1.2.3 Determination of ClO_2 concentration in water

ClO_2 and its major degradation by-product in water, ClO_2^- are both regulated species in drinking water due to the neurotoxic and other adverse health effects associated with ClO_2^-. The maximum contaminant level (MCL) of chlorite in drinking water is 1.0 mg/L whilst that of chlorine dioxide is 0.8 mg/L[17-18]. These are required to be monitored daily at the entrance of the drinking water distribution system according to Stage I of the Disinfection and Disinfectants By-products Rule (D/DBPR)[18]. The analysis of chlorine dioxide in water is complicated because inherently, it is volatile, decomposes upon exposure to light, unstable with time and may further be affected by interferences of other redox species such as free chlorine, nitrates, chloramines, and sulfates[2]. Several analytical techniques are employed for compliance monitoring or determining ClO_2 concentrations in water. Common methods generally applied for compliance monitoring are summarised in Table 2.1.

Table 2.1 Summary of common analytical methods for measuring the concentrations of ClO_2 and its oxychlorine by-products

Method	Brief Description	Strengths	Weaknesses	Reference
Iodometry	Involves a measure of the total available strength of the analyte solution to oxidise I^- to I_2	Suitable for standardizing ClO_2 solutions required for preparing temporary standards	Difficult to distinguish between fractions of Cl_2, ClO_2^-, and ClO_3^-	Standard Methods 4500-ClO_2-B[19]
Amperometric Titration I	Reactions of oxychlorine species with phenylarsine oxide by sequential titration at varying pH conditions and monitored by an electrochemical technique that measures current (A) at a constant voltage	Suitable for accurately distinguishing between the various oxychlorine species (Cl_2, ClO_2, ClO_2^-, and ClO_3^-)	-Interferences from organic chloramines such as dichloramines may lead to false positives as apparent ClO_2 residuals -Requires special equipment and skill -No longer recommended by the USEPA for analyzing ClO_2 in drinking water[2]	Standard Methods 4500-ClO_2-C[19]

Amperometric Titration II	Involves successive titrations of combinations of chlorine and oxychlorine species as described in Amperometric titration I above. However, complications associated with disproportionation at elevated pH is avoided	Suitable for accurately distinguishing between the various oxychlorine species (Cl_2, ClO_2, ClO_2^-, and ClO_3^-) It is still a recommended method by the USEPA	Requires special equipment and skill. May be subjected to interferences by Mn^{2+}, Cu^{2+}, and NO_2^- at low pH conditions	Standard methods 4500 - ClO_2-E[19]
N,N-diethyl-*p*-phenylenediamine (DPD)	This method involves either the titrimetric method where a solution of the analyte mixed with DPD acting as an indicator is titrated with Ferrous Ammonium Sulfate	Relatively more sensitive to low concentrations of ClO_2 can be detected	Positive interferences from ClO_2^- and free available chlorine species Currently not recommended by USEPA for compliance	

	(FAS) or the colourimetric procedure which entails the use of a spectrophotometer to measure the absorbance after mixing the analyte with DPD and comparing with a pre-determined standard curve.		monitoring in drinking water due to possible interferences from other oxidising species[2]	
Lissamine Green B (LGB)	Entails ClO_2 decolourizing LGB dye which is monitored by a spectrophotometer	Good sensitivity. Detection limit could be as low as 0.1mg/L Not subject to interferences from chlorine Can directly measure ClO_2 concentration	May suffer from errors of measuring by differences when used to examine ClO_2^- concentrations	USEPA method 327.1[20]
Chlorophenol Red	A reaction of ClO_2 bleaches CPR over a concentration	Detection limit could be as low as 0.1 mg/L		

(CPR) method	range 0.2 – 2.0 mg/L. The resulting decrease in colour is proportional to the concentration of ClO_2 and is monitored by a spectrophotometer at 574 nm	Fewer interferences from monochloramine, ClO_2^-, or ClO_3^- Requires minimal technical skill	[21]
Ion Chromatography	Involves the elution of the sample with Na_2CO_3 solution and further purging with $N_{2(g)}$ to dispel ClO_2	Suitable for measuring low concentrations of ClO_2^- and ClO_3^- as by-products of ClO_2 disinfection	Standard methods 4110-D [19]

2.1.2.4 Applications of ClO_2 in water treatment and industry

Chlorine dioxide was first applied as a water disinfectant to treat spa water in Ostend, Belgium in the early 1900s[13]. In the United States, the earliest use of ClO_2 at a treatment plant was at the Niagara Falls Water Treatment Plant (NFWTP) in New York in 1944 where it was applied to oxidise phenols to improve on odour[13]. In other parts of Europe, it was employed in water treatment mainly to oxidise phenols and to remove odourous chlorophenols which were associated with chlorine reactions with organic matter. At the later part of the 1970s when reports on the formation of trihalomethanes as disinfection by-products of chlorine emerged[22], the use of chlorine dioxide in drinking water treatment became popular. Particularly, this was so because chlorine dioxide was found not to chlorinate the organic precursors in the dissolved organic matter content of water to form the regulated DBP's such as trihalomethanes and haloacetic acids.

As a disinfectant, ClO_2 has been reported to possess impressive activity against bacteria[23-25], viruses[26-28] and protozoa[29]. It has been shown to be superior to chlorine in inactivating recalcitrant protozoan cysts such as *Cryptosporidium oocyst* and *Giardia lamblia* in water. Chlorine dioxide has been demonstrated to be effective at controlling biofilms attached to surfaces in water pipes[30] as well as Legionella sp[31-32]. Gaseous chlorine dioxide has effectively been applied as a fumigant to control microbial agents in building spaces and pests of food products[33-35]

Apart from the disinfection of microbial contaminants in water, it is also utilised for several other purposes in water treatment. Among these is the oxidation of soluble Fe^{2+} and Mn^{2+} in water. Applying chlorine dioxide as a pre-oxidant during treatment could effectively and rapidly oxidise and precipitate out the soluble forms of these metals[36] which could eventually be filtered out and thus prevented from entering the distribution system.

At neutral pH conditions where chlorine atom is completely reduced to Cl^-, approximately 0.24 mg/L of ClO_2 is required to oxidise 1.0 mg/L Fe^{2+} according to the stoichiometric equation below:

$$ClO_{2\,(l)} + 5Fe(OH)_{2\,(aq)} + 3H_2O \rightarrow Fe(OH)_{3\,(s)} + 10CO_{2\,(g)} + H^+_{(aq)} + Cl^-_{(aq)} \quad (16)$$

Meanwhile at pH > 8, where ClO_2^- is formed[2], about 1.2 mg/L of ClO_2 is consumed to oxidise 1.0 mg/L of Fe^{2+}

$$ClO_2 + Fe^{2+} + 3H_2O \rightarrow Fe(OH)_3 + ClO_2^- + 3H^+ \quad (17)$$

Similarly, stoichiometrically, 2.45 mg/L ClO_2 is required to oxidise 1.0 mg/L[4].

$$Mn^{2+} + 2ClO_2 + 4OH^- \rightarrow MnO_2 + 2ClO_2^- + 2H_2O \quad \text{at near neutral pH} \qquad (18)$$

$$Mn^{2+} + 2ClO_2 + 6H_2O \rightarrow 5MnO_2 + 12H^+ + 2Cl^- \quad \text{at below neutral pH} \qquad (19)$$

When these ions in treated water enter the distribution system, they may be precipitated by oxidation and adversely affect the taste and colour of the water, in addition to the staining of laundry and other household surfaces. Besides, the sediments of the precipitated metallic particles in the distribution system could also result in the clogging of pipes and a potential increase in energy loss in heating systems[4].

Off -taste and odour producing compounds such as chlorophenols and other phenolic compounds as well as mercaptans, dimethyl disulfide, dimethyl trisulfide and indole are also oxidised by ClO_2 in water[2]. This significantly improves the taste and odour of treated water. In recent times, considerable attention has been focussed on using ClO_2 to oxidise emerging micropollutants in wastewater effluents such as residues of pharmaceuticals and personal care products (PPCP) as well as endocrine disrupting agents that are resistant to degradation by conventional treatment methods[37-40]

Chlorine dioxide is also applied substantially in the pulp industry as a bleaching agent. It reacts with lignin in pulp in a manner that preserves pulp strength and produces clean, stable and high-brightness kraft pulp[41]. Due to the non-chlorinating characteristic of ClO_2, the discharges from the mill waste contain relatively lower adsorbable organic halides and dioxins[42] and that is positive for the aquatic lives of water bodies that receive the wastewater.

2.1.2.5 Limitations of ClO_2 as a disinfectant

Even though chlorine dioxide is an impressive disinfectant, there are some operational limitations to its application in practice. Chlorine dioxide is several times more expensive than chlorine. This limits its application in certain situations. It cannot be stored and transported as a compressed gas: it must rather be generated on site. The main by-products of chlorine dioxide disinfection are ClO_2^- and ClO_3^-. Currently, ClO_2^- is regulated and the regulatory limits prevent the application of high doses in drinking water treatment.

2.1.3 Ozone

2.1.3.1 Physical properties of ozone

Ozone (O_3) is a triatomic allotrope of oxygen. It is a pungent odourous and unstable colourless gas at room temperature but condenses to a dark blue liquid. It is usually present in a dilute form in a mixture of oxygen or air but concentrations exceeding 30 % in the gaseous form could be very unstable and explosive. Ozone is slightly denser (2.14 g/L) than air (1.28 g/L). It absorbs radiations in the infra-red, visible and ultraviolet wavelengths of the electromagnetic spectrum and has an absorption maximum at 253.7 nm [43]. Other selected physical properties of ozone are indicated in Table 2.2

Table 2.2 Selected physical properties of ozone

Property	Value
Molecular weight	48
Boiling point at 1 atm	-11.9 ± 0.3 °C
Melting point at 1 atm	-192.5 ± 0.4 °C
Critical temperature	-12.1 °C
Density at 0 °C	2.14 g/L
Solubility in water at (v/v) at 0 °C	0.64
Oxidising potential	-2.07 V
Latent heat of gas at boiling point and 1 atm	297 kJ/kg
Weight of liquid at boiling point	1352 kg/m^3

Source: [43]

2.1.3.2 Generation of ozone

Ozone exists naturally in the upper atmosphere of the earth to form the ozone layer which protects life on earth from harmful UV radiations. It could also be found in the lower atmosphere from the discharge of lightning during storms and other human activities.

Commercially, ozone is generated by two widely accepted approaches. These include; passing an oxygen-rich gas through a source of high energy electric field (corona discharge/ silent electrical discharge method) or by UV radiation (photochemical method)[44]. The corona discharge method which is employed in most commercial generators fundamentally involves the passage of oxygen-rich gas or ambient air through two electrodes of high energy electric field separated by a dielectric and a small discharge gap[2]. Oxygen molecules are dissociated by free energetic electrons into active radicals which readily combine with intact oxygen molecules to produce ozone.

$$O_2 + e^- \rightarrow 2O^{\cdot} \quad (20)$$

$$2O^{\cdot} + 2O_2 \rightarrow 2O_3 \quad (21)$$

The unstable O_3 can quickly revert to the more stable O_2 and for this reason, ozone is not stored and transported commercially but instead generated at the point of use.

$$O^{\cdot} + O_3 \rightarrow 2O_2 \quad (22)$$

On the other hand, the photochemical method rather utilises high energy UV radiations at a wavelength of 185 nm to split the oxygen molecules in the feed gas (usually ambient air) into highly reactive radicals which rapidly combine with the intact oxygen molecules to produce ozone.

Meanwhile, there are other generation methods such as electrolysis of sulphuric acid, radiochemical and reactions of elemental phosphorus with water, but these are economically expensive for industrial applications[44].

2.1.3.3 Solubility and Stability of ozone in water

The effectiveness of ozone as a disinfectant or abatement of micropollutants in water depends on its solubility and stability[44]. Ozone is sparingly soluble in water, but it is about 11.5 times more soluble than oxygen[2]. Consequently, a high ozone concentration could be obtained by bubbling an ozone/oxygen mixture from an oxygen-rich ozone generator. The solubility is also strongly dependent on temperature and as in the case of most gases; increasing the water temperature results in a reduction in the solubility of ozone. It has been suggested that the solubility at 0 ºC is twice as high as that of room temperature[44].

Aqueous solutions of ozone are inherently unstable, and this instability is further influenced by several contributing factors even though not all have been fully elucidated. Ozone is particularly unstable in basic solutions because of the formation of hydroxyl radicals (˙OH), from OH^- which are critical species for initiating and accelerating a radical chain decomposition reaction of ozone in water[45]. However, acidification of water and the addition of ˙OH radical scavengers such as bicarbonate could enhance the stability. Moreover, in natural and municipal wastewaters, stability is also largely dependent on the dissolved organic matter (DOM) content. Ozone readily reacts with the electron-rich aromatic components of the DOM which tend to increase the depletion rate whereas waters with low DOM and high bicarbonate content prolong the ozone life-time[44].

2.1.3.4 Ozone application for water treatment

When ozone is aerated into water, it undergoes reactions with the constituents' present. These reactions are primarily described by two reaction pathways: direct reactions with molecular ozone (O_3) or indirectly by ˙OH radicals generated from ozone decomposition. The details of the mechanisms involved in the free radicals facilitated ozone decomposition in water are described elsewhere[45-46]. Both O_3 and ˙OH are very strong oxidants and account for multiple reactions and applications in water.

Ozone may be applied in water for either disinfection, oxidation of both inorganic and organic compounds or particle removal. It has been demonstrated to exhibit rapid inactivation of bacteria such as *Legionella pneumophilia*[47] *Salmonella typhimurium*[48] and *Pseudomonas aeruginosa*[48], viruses[49-50], *Cryptosporidium parvum*[51-52] and *Giardia lamblia*[47] at relatively low concentrations. Ozone is also used extensively to oxidise emerging micropollutants such as

pharmaceuticals, pesticides and personal care products[53-54]. A pre-ozonation step during water treatment enhances coagulation and subsequent filtration of fine particles as well as micropollutants removal and to reduce the natural organic matter components that serve as suitable precursors for DBP of chlorination[55]. Ozone also improves on colour, oxidises algal toxins that may be present in surface waters and municipal wastewater. Furthermore, wastewaters treated with ozone are also well oxygenated and thus may not need any further aeration before discharging into receiving waters.

However, ozone reacts with Br^- ions to produce bromate (BrO_3^-) which is a regulated species in water due to its carcinogenic effects[56]. Another limitation is the inability to leave residuals in the distribution system and therefore the ozonated water may usually be followed by chlorination or chloramination to safeguard the treated water in the distribution system.

2.1.4 UV- Radiation

UV- light is a form of electromagnetic radiation found within the wavelength region of 100 – 400 nm. Four main classes of UV- radiations have been identified and these include: (a) UV-A: Ranging between 400 and 315 nm (b) UV-B: Ranging between 315 and 280 nm (c) UV-C: Ranging between 280 and 200 nm (d) UV- vacuum: Ranging between 200 and 100 nm.

Artificial UV radiation could be generated by a variety of lamps but the most widely used lamps for large-scale potable water applications are a low pressure (LP), low pressure-high-output (LPHO) and the medium pressure (MP) mercury vapour lamps. In recent times, other lamp technologies such as electrodeless mercury vapour lamps, pulsed UV lamps[57], and UV-LED[58] have shown significant promise.

UV- based disinfection results from the photo-biochemical changes that occur in the DNA or RNA of the target microorganism when it absorbs radiations of sufficient energy. The absorbed radiations predominantly promote changes such as thymine-thymine dimerization of the DNA nucleotides and uracil—uracil dimers of RNA which in turn inhibit the replication process of DNA synthesis and eventually rendering the organism inactive[59]. The optimum biocidal effect occurs within the UV-C region (200 – 280 nm) but the most potent biocidal activity occurs at 254 nm.

In water treatment, UV disinfection technology is broadly effective against non-spore forming bacteria, most viruses and the chlorination-resistant protozoa like *Cryptosporidium* and *Giardia* cysts. However, pathogens such as Adenovirus type 40 and 41, Norovirus and spores

of *Bacillus subtilis* and anaerobic cysts of *Clostridium perfringens* are less effectively inactivated by UV[60]. Disinfection by UV, unlike the chemical-based methods, does not generate disinfection by-products or leave any chemical residues that may affect the quality of treated water. The capital cost is also relatively cheaper than ozone and membrane filtration and does not require extensive technical expertise to monitor[2].

However, the absence of residuals in treated water samples is considered a major limitation to UV disinfection technology in potable water treatment because it is unable to offer protection for treated water in the distribution network[59]. Moreover, unlike other strong chemical disinfectants, it does not oxidise chemical impurities to improve the colour, taste, odour and the removal of iron and manganese. Also, the presence of particulate matter, colour, and turbidity adversely affect the transmission of UV radiations to the target microorganism resulting in considerable reduction in disinfection efficiency[61]. Consequently, UV disinfection is usually suitable for final treatment processes with reduced particulate matter and organics.

2.2 Fundamental theories of disinfection

Broadly, the processes of disinfection are pinned on two major competing concepts; the vitalistic and mechanistic theories. The vitalistic theory assumes that individuals in a pure culture of microorganisms possess different degrees of resistance towards a disinfectant and these characteristic differences within the population are permanent[62-63]. This concept best explains why individuals in a population of microorganisms are not destroyed at the same time by a disinfectant. Instead, whilst one group may be destroyed rapidly, others may require a longer time and sometimes higher doses of disinfectant to respond accordingly. However, this theory is unable to adequately explain why in most cases the more susceptible populations are invariably in the majority.

On the other hand, the mechanistic concept views the process of disinfection as an orderly time-process analogous to chemical reactions where the molecules of the disinfectant and the microorganisms are considered as the participating reagents[62-63]. This theory, unlike the vitalistic theory, assumes a general similarity of resistance among the individuals of a population. From the chemical reactions analogy and considering an excess of the disinfectant molecules, it follows that at any given time, only a proportion of the molecules of the reaction species take part in a series of unimolecular reactions that obey first-order kinetics. A limitation

of this theory is the inability to account for the existence of tailings which are usually observed with survival curves of inactivation.

2.3 Kinetic models of disinfection.

The process of disinfection is a complex phenomenon. Usually, the extent of inactivation achieved for a disinfection activity is influenced by multiple factors such as pH of the sample, disinfectant concentration, temperature of the reaction mixture and the vast diversity of microbial strains and complex structures[64]. Kinetic models thus simplify the complex phases of the reaction into mathematical expressions to enhance understanding of key influencing factors of the process. Moreover, the models and their underlying rate laws are useful and reliable basis for the design of disinfection criteria and evaluation of disinfection performance[65]. The engineering designs of contactor systems for disinfection also rely on empirical kinetic models.

A logarithmic relationship has been observed to exist between the concentration of surviving individuals in a reaction system and the contact time during disinfection. Consequently, the results of disinfection reactions are usually expressed as semi-log graphs called survival curves. Various types of microbial survival curves usually observed with disinfection kinetics are shown in Figure 2.1. The curve A indicates an exponential kill or first-order kinetics whilst B characterises reactions involving a rapid initial inactivation kinetics that is followed by a decrease in the rate to eventually produce a tailing. Such observations have been attributed to the existence of different subpopulations of organisms with varying resistance to a disinfectant[63, 65]. Curve C depicts an initial shoulder or lag phase preceding an exponential kinetics, and this has been suggested to be either due to insufficient mixing, a lag in the diffusion of disinfectant to the target sites in the organism or the occurrence of multiple inactivation sites within the organism[65] The curve D displays a shoulder phase, an exponential phase, and a tailing, normally observed with clumped organisms.

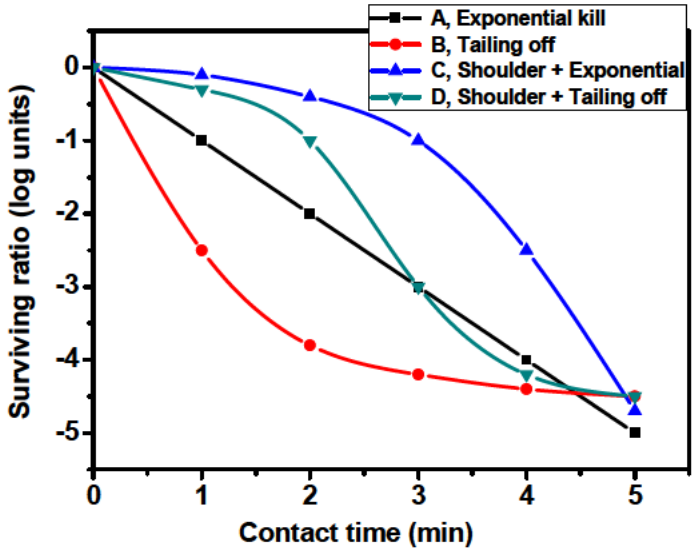

Figure 2.1. Typical microbial survival curves during disinfection.[65] A: indicates an exponential kill or first-order kinetics. B: shows reactions involving rapid initial inactivation kinetics, followed by a decrease in the rate thus resulting in a tailing-off. C: depicts an initial shoulder or lag phase preceding an exponential inactivation kinetics and D: displays an initial shoulder followed by an exponential phase and an eventual tailing-off.

Several models for water disinfection under both disinfectant demand-free and demand conditions have been advocated since the pioneering work of Chicks in 1908[66] but the most commonly used models include the Chick-Watson, Hom, Power law and Hom-Power law.

These models have been derived from the generalized differential rate law:

$$\frac{dN}{dt} = -k\, m\, N^{x}\, C^{n}\, T^{m-1} \qquad (23)$$

where $\frac{dN}{dt}$, is the inactivation rate, N is the number of surviving microbial organisms at contact time T; C is the disinfectant concentration and k is the inactivation rate constant which is determined experimentally. The parameters m, n and x are empirical constants. A brief review of some common models is discussed below.

2.3.1 Chick-Watson model

In 1908, Harriette Chick observed a similarity between an elementary bi-molecular chemical reaction and the reaction of a disinfectant with bacteria[66]. In the presence of excess disinfectant, the rate of the bacterial inactivation was expressed in what is known as the Chick's law

$$\mathbf{r} = \frac{dN}{dt} = -k'N \qquad (24)$$

where r is the inactivation rate and k' is *pseudo* first-order inactivation rate constant (time^{-1}). It states that at a constant disinfectant concentration, the rate of inactivation of a microorganism is proportional to the number of surviving microbes.

Subsequently, later in that same year, Watson[67] proposed an empirical log function establishing a relationship between k' and the concentration of the disinfectant as

$$k' = kC^n \qquad (25)$$

where k is a constant which is specific to a microorganism under a set of conditions (known as the *coefficient of specific lethality*), and n is the *coefficient of dilution* which is assumed to be the average number of disinfectant molecules that "react" with the organism to inactivate it. When this is incorporated into the Chick's law and integrated, a function which is now known as the Chick-Watson model is obtained as:

$$\ln \frac{N}{No} = -kC^nT \quad \text{or} \quad \log \frac{N}{No} = kC^nT \qquad (26)$$

where $\frac{N}{No}$ is the survival ratio of the microbes, with N and N_o indicating the number of microorganisms at time, t and 0 respectively. At $n = 1$, a simple Chick-Watson model which follows first-order kinetics is obtained. This suggests that an increase in the concentration of disinfectant and contact time in turn increase the inactivation of microorganisms. The model is simple and more attractive for the design of contactor systems and regulatory practice. For instance, the CT value derived from the Chick-Watson kinetics as the product of the disinfectant concentration, C and the contact time, T required to achieve 99 % inactivation is a widely utilised tool to evaluate the efficiency of a disinfectant on a target microorganism under a set of conditions. The model however, does not adequately describe inactivation kinetics which deviate from first-order kinetics such as survival curves displaying shoulders or tailings.

2.3.2 Hom model

This model was first advanced by Hom in 1972[68] to account for the observed curvilinear relationship between the survival ratio of algal-bacterial exposed to chlorine and contact time. It makes an empirical generalization of the *pseudo* first order Chick-Watson rate law

$$\frac{dN}{dt} = -k\,\mathrm{mNC}^{n}\,\mathrm{T}^{m-1} \qquad (27)$$

The integral of equation 27 yields the empirical Hom model

$$\ln\frac{N}{No} = -\,k\mathrm{C}^{n}\mathrm{T}^{m} \quad \text{or} \quad \log\frac{N}{No} = k\mathrm{C}^{n}\mathrm{T}^{m} \qquad (28)$$

Generally, the level of inactivation predicted by the Hom model is not a linear function of C and T but rather dependent on the empirical model parameters *m* and *n*. When m = 1, the model simplifies to the Chick-Watson model, otherwise a curve with a shoulder is observed when m >1 whilst a tailing is displayed at m < 1.[65] This suggests that the original Hom model could either indicate a tailing or a shoulder and not both simultaneously. A modified form of the Hom model has been proposed to simultaneously describe a survival curve characterised by a shoulder, log-linear and a tailing[69]

$$\log\frac{N}{No} = k_1[1\text{-}\exp(k_2 t]^{k3} \qquad (29)$$

where k_1, k_2, and k_3 are empirical constants of the model representing the shoulder, log-linear and tailing regions of the survival curves respectively.

Several forms of the Hom model have been derived to suitably describe inactivation kinetics under diverse conditions such as disinfectant decay. A summary of such Hom-based models is shown in Table 2.3.

Table 2.3 Inactivation kinetic models derived from the empirical Hom model under disinfectant decay conditions

Model	Survival ratio (log units)	References
Integral Hom	$-\mathbf{k}mC_o^n \sum_{k=1}^{z}(e^{-km})_k t_k^{m-1}\Delta t_k$	70
Efficiency Hom	$-\mathbf{k}C_o^n T^m \eta$ $\eta = \left[\frac{1-exp(-nk_dT)}{nk_dT/m}\right]^m$	71
C_{avg} Hom	$-\mathbf{k}C^n_{avg} T^m$ $C_{avg} = \sqrt{C_o.C_f}$	65
Incomplete gamma Hom	$\frac{kmC_o^n}{(nk')^n}$, γ (m, nk´T)	71

2.3.4 Power law (Rational) model

The power law kinetic expression is the general rate law at m =1

$$\frac{dN}{dt} = -k\, N^x C^n \qquad (30)$$

Integration of the rate law (equation 30) yields the Rational model[65, 72] as:

$$\log\frac{N}{No} = \frac{-log\left[1+N_0^{x-1}(x-1)\right]kC^nT}{X-1} \qquad x \neq 1 \qquad (31)$$

The model has an additional independent variable N_o which represents the initial microbial density in the reaction system. For an inactivation kinetics where $x < 1$, a shoulder is

observed whilst a tailing off behaviour results at $x > 1$. A modified form of the rational model which accounts for a first-order disinfectant decay has also been derived as:

$$\log \frac{N}{No} = \frac{-log[1+N_0^{x-1}(x-1)]\frac{k}{k'n}(C_0^n - C_f^n)}{X-1} \qquad x \neq 1 \qquad (32)$$

where k' is the first-order decay rate constant of disinfectant and C_o and C_f are the initial and final concentrations of disinfectant respectively.

2.3.5 The Hom-Power law model

This model was developed by Anotai[73] to incorporate all the parameters of the Hom model and the Power law model as contained in the generalized differential rate law. Under disinfectant demand free conditions in a batch reactor system, the integral form of the model can be expressed as;

$$\log \frac{N}{No} = \frac{-log[1+N_0^{x-1}(x-1)]kC^nT^m}{X-1} \qquad (33)$$

The parameters k, m, n, and x maintain their meanings as in the individual models. It provides a better fit or prediction than either the Hom model or the Power law model for survival curves showing shoulders or tailings. However, the high number of parameters limit its application in practice and should only be applied as a best-fit model when there is a significant improvement over relatively simple models.

Other differential models which provide suitable explanations and fittings to survival curves have also been developed to account for shoulders, tailings, and sigmoidal shaped graphs. Some of these include the Selleck model[65], Series Event model[74-75], Multiple Target models[65] and Intrinsic quenching model[76]

2.4 Bacterial Cell Structure

A typical vegetative bacterial cell (Figure 2.2) consists of a chemically complex external layer called the cell envelope that lies outside the cytoplasm. The cell envelope is made up of two layers: the outer cell wall and the inner cell membrane which are only separated by the periplasmic space but are essentially stacked together and function to maintain cell integrity[77].

The cell membrane, also known as the plasma membrane or cytoplasmic membrane is a very thin (5-10 nm) flexible sheet phospholipid bilayer embedded with various globular sized proteins and encloses the cytoplasm[78]. It is selectively permeable and regulates the transport of molecules and nutrients into the cytoplasm as well as the discharge of metabolic products into the extracellular environment. Most enzymes involved in energy generation reactions of respiration and the synthesis of structural macromolecules for the cell wall reside in the cell membrane.

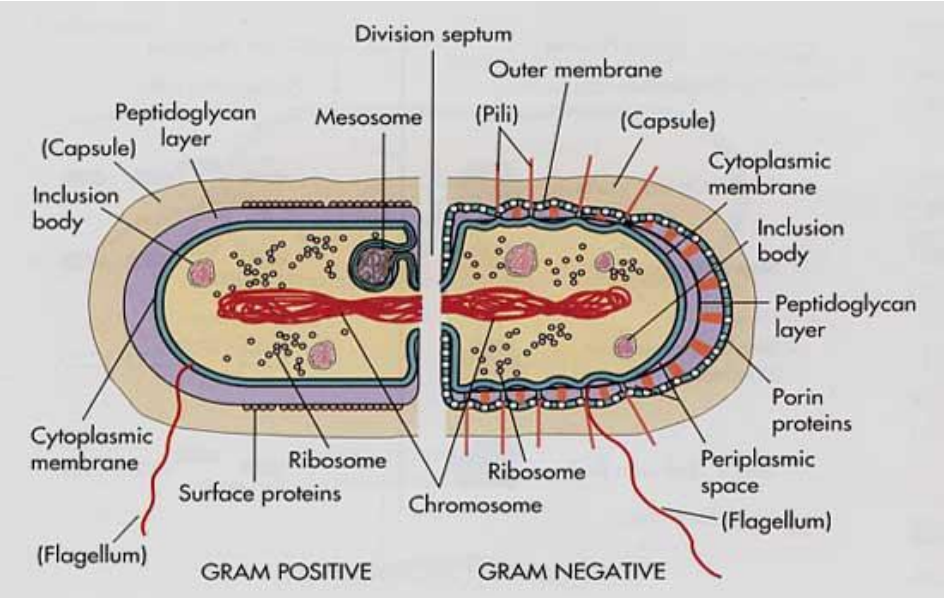

Figure 2.2 A typical vegetative bacteria cell[79]

The cytoplasm is a ‘complex pool’ of water (70% - 80%) and a prominent site for most of the cell’s biochemical and synthetic activities including protein synthesis[80]. The matrix also contains sugars, salts, amino acids other organic molecules which are building units of cell

synthesis and source of energy. Other discrete components such as chromosomes, inclusion bodies, and ribosomes are also located in the cytoplasm.

The cell wall is a fairly rigid layer that encloses the cytoplasmic membrane[80]. It gives the cell's shape and provides support and protection from osmotic lysis. The relative strength and robust nature of the cell wall emanate from a unique macromolecule: the peptidoglycan. Many pathogenic strains of bacteria have their pathogenic components located in the cell wall. For instance, in Gram-negative bacteria, lipid endotoxins in the cell wall stimulate shock reactions that lead to infections such as meningitis and typhoid fever whilst surface proteins attached to Gram-positive strains including *Corynebacterium diphtheriae* (the agent of diphtheria) and *Streptococcus pyogenes* (causes sore throat) possess toxic properties. External stress conditions such as toxins are resisted by the cell wall, and hence it becomes the target site for most antibiotics and biocides[81].

2.4.1 Gram-positive and Gram-negative cells

In 1884, Hans Christian Gram developed the Gram stain that provided the basis for delineating bacteria into two major groups based on their responses to the staining procedure. Gram-positive bacteria retain the stain to produce a purple colour whilst the gram-negatives do not and are coloured pink or red. However, it is now established that the underlying factor of this difference lies with the structural differences in their cell wall[77].

The Gram-positive cell walls are primarily made of thick (20 – 80 nm thick) homogeneous layer of peptidoglycan[82]. A large amount of teichoic acids which are anionic polymers of glycerol or ribitol and phosphate groups are embedded in the peptidoglycan sheath and may extend to the surfaces. Due to the negatively charged nature of teichoic acid molecules, the gram-positive cells possess a net negative charge[83]. Most species also have surface proteins attached to the outer surfaces of the peptidoglycan and are involved in interactions with the external environment of the cell.

Gram-negative bacterial cell wall is relatively more complex morphologically[80]. It is made up an outer membrane (OM) and a thinner sheath of peptidoglycan beneath it. The OM contains lipopolysaccharides (LPS) which are large, complex molecules containing lipids and

carbohydrates that enhance the stability, strength and the structural integrity of the membrane. Endotoxic shocks commonly associated with infections of Gram-negative bacteria are linked to the lipid A component of the LPS molecule[84].

There are also porin proteins embedded in the OM and these are hydrophilic channels that allow the passage of small molecules like glucose and other monosaccharides whose molecular sizes are less than about 600 to 700 daltons whilst excluding larger molecules. The OM provides an extra protective barrier to the gram-negative bacteria against the entry of substances such as bile salts, antibiotics and large molecules of disinfectants or antimicrobials[85]. In general, the OM in Gram-negative bacteria acting as a permeability barrier reduces the uptake of biocides and consequently becomes more resistant to antibiotics and disinfectants than the gram-positive strains.

2.4.2 *Escherichia coli*

Escherichia coli (*E. coli*) is a Gram-negative facultative anaerobic bacillus and non-sporulating bacterium. It is a member of the family *Enterobacteriaceae* and commonly found in the gastrointestinal tracts of warm-blooded organisms. Most serotypes of *E. coli* exist as part of the normal microflora of the gastrointestinal tract and are harmless. They even provide some benefits to the host like preventing the colonization of the gut by pathogenic organisms[86]. However, some few strains have evolved and developed pathogenic strategies to cause diseases such as diarrhea, hemorrhagic colitis, hemolytic uremic syndrome, urinary tract infection and neonatal meningitis in humans[87]. Six main pathotypes of enteric *E. coli* infections based on their pathogenicity profiles have been identified. These include Enterohemorrhagic or Shiga toxicogenic *E. coli* (EHEC/STEC), Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC), Enterotoxigenic *E. coli* (ETEC) and Diffusely Adherent *E. coli* (DAEC). In humans, the infections of the enteric *E. coli* are generally through consumption of contaminated water or food[88].

In water, the presence of *E. coli* is a strong indication of a recent sewage or animal waste contamination. It is thus used as an appropriate indicator by regulatory agencies to predict the potential presence of enteropathogenic serotypes of bacteria, viruses or protozoa such as *Salmonella typhi*, *Salmonella paratyphi*, *Shigella dysenteriae*, *Vibrio cholerae* and *Cryptosporidium* oocyst from faecal contamination[88] and to assess the quality of potable water treatment.

2.4.3 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (*P. aeruginosa*) is a Gram-negative, facultative anaerobe rod-shaped bacterium that belongs to the family Pseudomonadaceae[89]. It is ubiquitous in the environment and can be found in faeces, sewage, soil, water and other moist and nutrient-limited environments like sinks, water baths, showers, spa pools and hot water systems[88]. It is often described as an opportunistic pathogen due to its association with life-threatening ailments in burn and surgical patients and in immunocompromised patients in hospital environments and also a common cause of infections in patients suffering from cystic fibrosis[90] . Higher numbers in potable water, most especially packaged water have notably been associated with taste, odour and turbidity complaints.

Several studies have linked nosocomial *P. aeruginosa* outbreaks to hospital water sources[91-93] and therefore their control in hospital water systems is now very vital in any risk management strategies in a hospital setting. Many strains have developed intrinsic machinery that enhances resistance towards a range of antimicrobial agents[94].

2.4.4 *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) is an aerobic or anaerobic, non-motile, non-sporulating Gram-positive cocci-shaped bacterium of the *Staphylococcaceae* family[95]. It is relatively widespread in the environment but frequently found in the mucous membranes and skins of animals. Most strains of *S. aureus* are generally non-pathogenic, but some few others could cause diseases like boils, skin sepsis, post-surgical wound infections and pneumonia. The methicillin-resistant *S. aureus* (MRSA) strains have particularly gained considerable attention in recent times owing to the risks of transmitting potential infections from animals to humans [96]. *S. aureus* has been cited as the leading cause of foodborne infections presently[97].

S. aureus can be released into water by human contact into water environments such as swimming pools, spa pools, and recreational waters.

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CHAPTER 3

Chlorine dioxide oxidation of *Escherichia coli* in water - A study of the disinfection kinetics and mechanism

Abstract

This study investigated the kinetics and mechanism of chlorine dioxide (ClO_2) inactivation of a Gram-negative bacteria *Escherichia coli* (ATCC 35218) in oxidant demand free (ODF) water in detail as a function of disinfectant concentration (0.5 – 5.0 mg/L), water pH (6.5-8.5), temperature variations (4 – 37 ºC) and bacterial density (10^5 - 10^7 cfu/mL). The effects of ClO_2 on bacterial cell morphology, outer membrane permeability, cytoplasmic membrane disruption and intracellular enzymatic activity were also studied to elucidate the mechanism of action on the cells. Increasing temperature and disinfectant concentration were proportional to the rate of cell killing, but efficacy was found to be significantly subdued at 0.5 mg/L and less dependent on the bacterial density. The bactericidal efficiency was higher at alkaline pH of 8 or above as compared to neutral and slightly acidic pH of 7 and 6.5 respectively. The disinfection kinetic curves followed a biphasic pattern of rapid inactivation within the initial 2 min which were followed by a tailing even in the presence of residual biocide. The curves were adequately described by the C_{avg} Hom model. Transmission Electron Microscopy images of the bacteria cells exposed to lethal concentrations of ClO_2 indicated very little observable morphological damage to the outer membranes of the cells. ClO_2, however, was found to increase the permeability of the outer and cytoplasmic membranes leading to the leakage of membrane components such as 260 nm absorbing materials and inhibiting the activity of the intracellular enzyme β-D-galactosidase. It is suggested that the disruption of the cytoplasmic membrane and subsequent efflux of intracellular components result in the inactivation of the Gram-negative bacteria.

Keywords: *Escherichia coli; Pseudomonas aeruginosa;* Chlorine dioxide; Disinfection; Kinetics.

3.1 Introduction

The reactions of chlorine with Natural Organic Materials (NOM) in water have been found to result in the formation of harmful disinfection by-products (DBP) such as trihalomethanes (THM), haloacetic acids (HAA), and haloacetonitriles (HAN)[1-2]. Subsequent epidemiological and toxicological studies have linked these DBP of chlorination in water to bladder cancer as well as adverse reproductive and developmental effects including early term miscarriages and stillbirths among pregnant women[3-4]. Moreover, there are also new challenges with the emergence of waterborne pathogens like *Giardia* and *Cryptosporidium*, which have been shown to be resistant to conventional chlorination[5-6]. Due to these concerns, regulatory bodies in the water treatment industry have placed emphasis on the use of alternative disinfectants in the treatment process to control the deleterious effects of the DPB whilst simultaneously maintaining adequate control of targeted pathogens[7].

Chlorine dioxide (ClO_2) is considered as a very useful and attractive alternative disinfection technology to chlorination[8-9]. It is neutral, volatile and highly energetic monomeric free radical that has high solubility in water. Contrary to the hydrolysis of chlorine gas in an aqueous solution, ClO_2 does not hydrolyze to any appreciable extent but remains in a solution as a dissolved gas[10]. ClO_2 is a highly effective oxidant by virtue of its one electron transfer mechanism, where it is primarily reduced to chlorite (ClO_2^-)[11] but it does not chlorinate the humic substances that may be contained in water from natural sources to form the deleterious volatile and non-volatile organic compounds like THM and HAA[12]. Apart from its excellent disinfection capacity, it also controls odour and colour challenges associated with water treatment by oxidising iron, manganese, and phenols[9]. Limitations of ClO_2 as a water disinfectant is primarily associated with the formation of inorganic DBP, chlorite (ClO_2^-) and chlorate (ClO_3^-) which are also regulated contaminants in potable water[11]. Nonetheless, its strong oxidation capabilities still make it a very useful and attractive alternative to chlorine.

Several studies in literature have previously demonstrated the effectiveness of chlorine dioxide as a disinfectant against bacteria,[13-14] viruses[15-16] and chlorination resistant protozoa such as *Giardia* and *Cryptosporidium*[17-18]. However, the application of reliable microbial inactivation laws and kinetic models in designing appropriate disinfection criteria at the water treatment plant is vital to the effective control of pathogenic microorganisms in potable water capable of safeguarding public health. The first order Chick-Watson model has been used to adequately

describe the inactivation kinetics of chlorine in oxidant demand free waters[19-20]. On the contrary, the survival curves of organisms exposed to ClO_2 have been characterised by a biphasic pattern; with a rapid initial phase, followed by a tailing off behaviour[15-16, 21] which indicate a deviation from the first order kinetic plot. Meanwhile, the extent of influence of other disinfection control parameters such as temperature, pH of the water, organic matter content or particles of attachment by the organism, type of organism and aggregation on the inactivation kinetics of ClO_2 unlike free chlorine has not been well characterised.

Moreover, the exact bactericidal mechanism of ClO_2 is still not clear and the limited information available is also contradictory. For instance, in a pioneering work of its mechanism on *Escherichia coli,* Bernade et al[13] observed that the primary lethal lesion of ClO_2 involved the disruption of the protein synthesis mechanism of bacterial cells rather than the inactivation of an enzyme system in the catabolism of glucose, as suggested in an earlier study involving chlorine[22]. In a later study, however; the primary lethal effect of ClO_2 was found not to lie with the disruption of protein synthesis or DNA inactivation[23]. A more recent study[24] observed some level of damage to cell surface membrane and degradation of inner cellular components of *E. coli*. Meanwhile, elucidating the principles of interaction of chemical biocides with organisms provides scientific bases for the optimization of activity and an understanding of how organisms circumvent this activity. It also helps to make predictions about the enhancement of synergism when disinfectants are applied in combinations. This is particularly important in an era of increasing challenges of antibiotic-resistant microorganisms and reports of disinfectant facilitated selection of resistant antibiotic strains in waste water[25-26].

In this study, we, therefore, monitored the kinetics of ClO_2 disinfection of a Gram-negative bacteria, *E. coli* spiked into an oxidant demand free laboratory water in a batch reactor as a function of disinfectant dosage, water temperature, pH and bacterial cell density. We further examined its efficacy in municipal wastewater samples. In addition, the bactericidal mechanism of action of ClO_2 on the Gram-negative bacteria was also investigated by studying its disruptive effect on the outer cell membrane permeability, cytoplasmic membrane integrity, and intracellular enzymatic activity as well as changes in cell morphology.

3.2 Materials and methods

3.2.1 Microbial strains and preparation of bacterial suspensions

A culture collection strain of bacteria was employed in this study: *Escherichia coli* (ATCC 35218) was obtained from the Microbiology Discipline, University of KwaZulu-Natal, Westville. Influent wastewater samples were collected from the EThekwini Municipal Waste Water Treatment Plant, Durban. The preparation of microbial suspensions for disinfection is described in the Supporting Information (Text 3.1, page 66).

3.2.2 Chlorine dioxide preparation and measurement

A stock solution of ClO_2 was prepared by oxidising about 25% (w/v) sodium chlorite ($NaClO_2$) by slowly adding a dilute solution of sulphuric acid (H_2SO_4, 2M) to the $NaClO_2$ solution. [27] The concentration of ClO_2 stock solutions was determined by the iodometric method whilst the residual concentrations were analysed by the DPD colourimetric method[27].

3.2.3 Disinfection experimental procedure

The disinfection experiments were performed in a 0.1 M PBS (pH 7.4) solution using oxidant demand free (ODF) deionized water. Oxidant demand free water was employed in the study to obtain uniform reproducible water quality that could be reproduced on demand[17]. Glassware were also treated to be oxidant demand free by soaking in at least 10 mg/L ClO_2 solution for 4 h and rinsed several times with deionized water. A glass container fitted with a lid and wrapped with an aluminum foil to minimise volatilization and photodecomposition of the ClO_2 was used as a batch reactor containing 500 mL of test solution undergoing agitation by a Teflon coated magnetic stir bar and the suspension in the reactor allowed to equilibrate at the temperature at which measurement was made. A calculated dosage of ClO_2 from the working solution was then added to initiate the reaction. Five milliliters (5 mL) of samples were collected from the sampling port of the reactor at various time intervals of 0.5, 1.0, 2.0, 5.0, 30.0 and 60.0 min using a 10 mL sterile syringe into sampling bottles containing excess sodium thiosulphate ($Na_2S_2O_3$) to instantaneously quench the residual disinfectant[24]. Preliminary experiments with sodium thiosulphate (data not shown) did not indicate any significant effect on the survival of the bacteria. Bacterial populations were enumerated after appropriate dilutions using the spread

plate technique[28] on a nutrient agar (Merck, South Africa) and incubated at 37 ºC for 24 h. Similarly, the inactivation of bacteria in the wastewater samples (pH 6.85-7.2, COD; 93.32-129.5 mg/L, SS; 5.6-7.9 mg/L and viable bacteria density 1.3 x 10^3- 2 $\times 10^5$ cfu/mL) were carried out as described above.

3.2.4 Effect of water temperature and pH on the ClO_2 disinfection

The effect of water temperature on the kinetics of ClO_2 disinfection of the bacteria was investigated by carrying out the above disinfection procedure at temperatures 4, 15, 22, 30 and 37 with ± 2 ºC standard deviation at pH 8.05 and applying 1.0 mg/L ClO_2. In each case, the bacterial suspension in the reactor was allowed to equilibrate with the surrounding temperature before the disinfection reaction was initiated. Series of disinfection experiments as described in Section 3.2.3 were conducted at 22 ± 2 ºC by varying the buffered water within the pH range of 6.5-9, which is usually encountered in natural waters.

3.2.5 Effect of bacterial density on the rate of inactivation.

To obtain different initial densities of the bacterial cells, fresh overnight cultures grown in Lysogeny broth (LB) (1-2 $\times 10^8$ cfu/mL) were centrifuged, washed twice in PBS (pH 8.05) and suspended in 100 ml of sterilised ODF buffered water at the same pH and stirred with a magnetic stirrer for uniform mixing. Subsequently, 1.0 mL portions of the stock suspensions were serially diluted to obtain approximate cell densities of 10^7, 10^6 and 10^5 cfu/mL in 100 mL of the reaction matrix in 250 mL Erlenmeyer flasks all under aseptic conditions. ClO_2 initial concentration of 0.75 mg/L was applied to each of the *E. coli* suspensions at a temperature of 22 ± 2 ºC and the rates of inactivation were monitored. All experiments were conducted in triplicates.

3.2.6 Mechanism of action of ClO_2 on bacteria.

The inactivation mechanism of ClO_2 was investigated by monitoring its effect on cell outer membrane permeability, cytoplasmic membrane integrity and its consequent effect on intracellular enzymatic activity as well as morphological changes.

3.2.6.1 Cell outer membrane permeability.

The outer cell membrane permeability activity of ClO_2 was examined by the 1-N-phenyl naphthylamine (NPN) uptake assay as described previously[29-30]. Briefly, a stock solution of 10 mM NPN was prepared by dissolving in acetone. Bacteria grown to a mid-logarithmic phase (OD_{600} value of 0.5 ± 0.02) were centrifuged at 6000 × *g* for 10 min, washed twice in HEPES (N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid) buffer (pH 7.4) and suspended into half volume of 5 mM of the same buffer. ClO_2 was then added at an adjusted dosage to give at least 2-log inactivation of cells after which it was quenched with 0.1 M sodium thiosulphate solution. This process was necessary because preliminary trials showed that the presence of ClO_2 interfered with the NPN fluorescence. Consequently**,** a similar amount of sodium thiosulphate was added to all other controls. NPN was added to the cell suspension to a final concentration of 10 µM in the HEPES buffer and the fluorescence intensity measured within 5 min with a fluorescence Spectrometer (Perkin Elmer LS 55) at a slit width of 5.0 nm and excitation and emission wavelengths of 350 nm and 420 nm respectively using 10 mm quartz cuvette.

3.2.6.2 Disruption of cytoplasmic membrane integrity.

This was investigated by monitoring the release of cytoplasmic components such as 260 nm absorbing materials and the accessibility of intracellular enzyme β-D-galactosidase to its substrate o-nitrophenyl-β-D-galactopyranoside (ONPG) [24]. This was related to both the impact on cytoplasmic membrane permeability and enzyme inactivation.

3.2.6.2.1 Kinetics of β-D- galactosidase release.

A procedure as previously described by Cho et al,[24] with slight modification was adopted in the ONPG assay using *E. coli* (Supporting Information Text S3.2, page 66). All experiments were conducted in triplicates.

3.2.6.2.2 Measurement of 260 nm absorbing materials.

Bacterial cultures grown in LB for 24 h were harvested at 4 ºC by centrifuging at 5000 *x g* for 10 min, washed twice in sterile PBS (10 mM, pH 7.4) and suspended in fresh 10 mL portions of the same buffer. Batches of cell suspensions (10^8 cfu/mL) were treated with different concentrations of ClO_2 (0.5, 1.0, 2.5 and 5.0 mg/L) except for the control. Two mL samples

were removed from the reactor at 5 min intervals and centrifuged immediately to separate the bacteria cells and the clear supernatants. The optical densities of the supernatants were then determined at 260 nm using a NanoDrop 2000C spectrophotometer (Thermo Scientific). Preliminary trials showed that the use of sodium thiosulphate solution to quench the ClO_2 reaction interfered with the absorbance measurements taken at 260 nm since the quenching agent also absorbs at a very close wavelength (254 nm) and hence that activity was avoided. The time points used here, therefore, indicate the period of contact allowed until the cells were separated from the supernatants. Absorbance measurements of ClO_2 samples only in the buffer were also determined at 260 nm.

3.2.6.3 Transmission electron microscopy (TEM) analysis.

TEM (JEM -2100 JEOL, Japan) was used to analyze the effect of ClO_2 on the morphology of the cells. Detailed procedure is described in the Supporting Information (Text S3.3, page 67).

3.2.7 Kinetic data analysis.

The inactivation survival data of *E. coli* was adequately described by the C_{avg} Hom (C_aH) model [19]. The Hom model has previously been found to best describe inactivation survival data of a wide range of disinfectants and microorganisms in a variety of conditions mostly characterised by a tailing off behaviour[15, 31]. The model is expressed as:

$$\log (N_t / N_0) = -kC^n_{avg} T^m \quad (1)$$

$$C_{avg} = \sqrt{C_0 . C_f} \quad (2)$$

where C_0 and C_f are the initial and final disinfectant concentrations (mg/L) respectively, (N_t/N_0) is the survival ratio of number of organisms surviving at time, t (N_t) and at time, t = 0, N_0 (cfu/ml)), *k* is inactivation rate constant of the target organism, T is the contact time required to achieve a given level of inactivation, *n* is an empirical factor called the *coefficient of dilution,* whilst *m* is an empirical constant. In this model unlike the simple Chick -Watson, the level of inactivation is not a linear function of C and T, but dependent on the model parameters *n* and *m* respectively which do not have values of unity. The value of $n < 1$ is an indication that the disinfection process is relatively more sensitive to contact time than the concentration. On the other hand, a higher value of *n* implies, the microbiocidal activity of the disinfectant is more dependent on the concentration than on contact time and hence effectiveness significantly decreases with dilution [31]. When $m < 1$, the survival curve produces a tailing effect, whilst an

initial shoulder is observed when $m > 1$[19, 32]. The Microsoft Excel 2013 Solver function was used to minimise the sum of squares of the difference between the experimentally observed and the calculated survival ratio based on the C_aH model to determine the optimal values of the parameters *k*, *m*, and *n*. The student *t*-test was used to assess the statistically significant differences ($p \leq 0.05$) of the effect of an experimental condition on the bacterial survival data.

3.3 Results and discussion

3.3.1 Disinfection kinetics of ClO_2 on *Escherichia coli*

The inactivation kinetics of *E. coli* exposed to different concentrations of ClO_2 (0.5, 1.0, 2.5, 3.5, and 5.0 mg/L) at 22 ± 2 ºC and pH 8.0 are illustrated in Figure 3.1. The kinetic parameters of the experimental data obtained at different concentrations and fitted to the C_{avg} Hom model are summarized in Supporting Information (Table S3.1, page 68) The level of inactivation achieved for the different concentrations as a function of time were all found to be more dependent on disinfectant dose and less at the expense of time. For instance, Figure 3.1 shows that under similar experimental conditions, 0.5 mg/L of ClO_2 exposed to *E. coli* for 60 min produced less than 1-$\log_{10}$ (90%) inactivation. This is approximately 30 mg min/L of CT (product of the disinfectant concentration, C in mg/L and the contact time, T in minutes) credit. However, an increase of the concentration to 1.0 mg/L resulted in 4.05 $\log_{10}$ (99.99%) inactivation of *E. coli* in just 5 min corresponding to a CT credit of 5 mg min/L.

A first-order kinetic plot of $\log_{10}$ of death time required for 2-log (99%) inactivation vs log of concentration from the survival data showed that the *dilution coefficient n*, of ClO_2, has an approximate value of 2.5. This suggests that the microbiocidal activity is more sensitive to the concentration applied as compared to the contact time and thus the effectiveness of ClO_2 substantially decreases with dilution[31]. The survival curves demonstrate a biphasic pattern of inactivation; with a quick initial kinetics phase preceding a slower kinetics or tailing effect. In the experimental data obtained for this study, the level of inactivation achieved at a given dose occurred mostly within the initial two minutes of exposure followed by a tailing which does not indicate any significant difference ($p > 0,05$) between t = 2 and 60 min. These results agree with similar studies involving the use of ClO_2 in inactivating microbes in water[13-14, 16].

Similarly, this pattern was observed in the inactivation of the bacterial population in the wastewater samples (Supporting Information, Figure S3.1, page 68). However, the activity was comparatively lower, and this could probably be due to interferences from the oxidant demand components in the wastewater samples. [33]

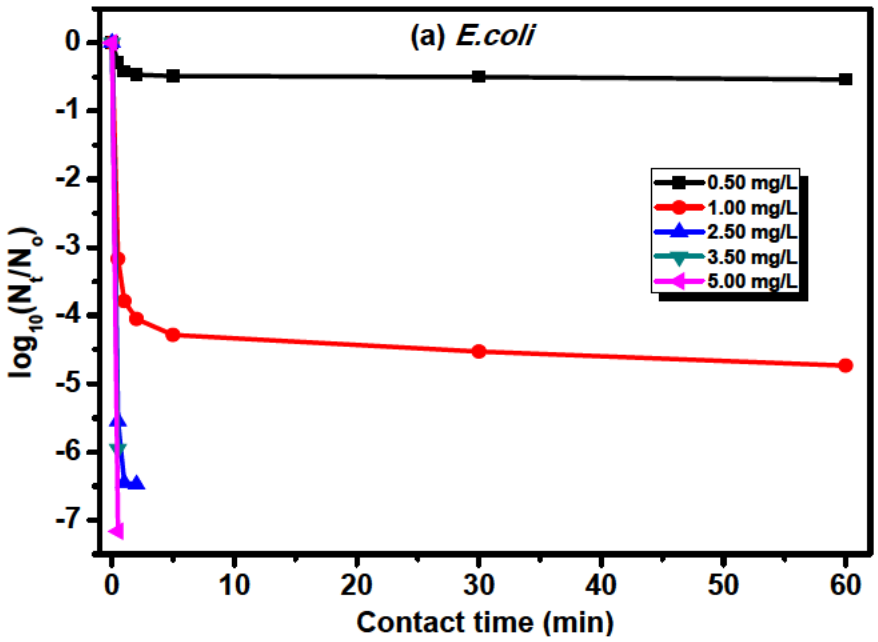

Figure 3. 1. Inactivation survival curves at different ClO_2 concentrations at 22 ± 2 ºC, pH 8.0

The tailing phenomena in the disinfection survival curves reported in other studies have basically been attributed to a number of factors including microbial aggregation leading to incomplete exposure, the presence of resistant microbial sub-populations as a result of population heterogeneity, decay in disinfectant concentration over time, hence diminishing efficiency and changes in the condition of the disinfection medium[34-35]. An experimental set-up to monitor the changes in ClO_2 concentration due to consumption or evaporation within the period of the experiments (data not shown) did not show any significant decrease in the concentration that could account for such a marked decrease in the inactivation rate resulting in a tailing. Moreover, the disinfection experiments carried out were designed to eliminate clumping of cells by adequate vortex mixing after centrifuging and to maintain other physical

parameters constant. Further investigations were carried out to establish whether the presence of the resistant bacterial sub-populations is responsible for the slow inactivation at the tailing phase following a similar method described by Hornstra et al[15] as follows.

An initial suspension of *E. coli* (~10^7 cfu/mL) was exposed to approximately 0.75 mg/L ClO_2, enough to give just 2-log inactivation at the tailing phase after 30 min under similar disinfection conditions as described earlier in ODF water. Forty milliliters samples were collected from the reactor and quenched with 5 ml of 0.1M sodium thiosulphate in 50 mL centrifuge tubes. Samples were then pelleted at 5000 *x g* for 5 min, washed thrice in PBS and suspended into fresh amount of sterile ODF water in a reactor under similar experimental conditions and exposed to 0.75 mg/L of the disinfectant. A similar pattern of inactivation curve was obtained with tailings observed after a rapid 2-log of inactivation. It would have been expected that the resistant populations would resist further inactivation by the same disinfectant at almost the same concentration under similar conditions and result in an initial slow inactivation.

Meanwhile, when a higher ClO_2 concentration (2.0 mg/L) was applied to a similar initial population, almost all bacteria were inactivated with no tailings in just 30s. The results herein, however, do not conclusively exclude the presence of resistant sub-populations as one of the causes of tailings in ClO_2 disinfection of the Gram-negative bacteria. Hence, a more comprehensive study may be required to draw such a conclusion. This observation rather re-emphasises the need to apply an adequate dosage of ClO_2 to achieve a desired level of inactivation irrespective of the contact time allowed. ClO_2 has been observed to exhibit self-limiting disinfection effect which inhibits further disinfection in viruses with time and has been suggested to be the main cause of tailings involved in its disinfection process[34].

3.3.2 Effect of temperature.

The experimental results obtained for the dependence of the rate of ClO_2 inactivation of *E. coli* on temperature are as summarised in Table 3.1. The rate constant for each temperature data set was obtained by fitting the survival curves to the non-linear C_aH model and the variations of the rate constant *k* with absolute temperature (T) in Kelvin were described by the Arrhenius equation:

$$k = \mathrm{A}e^{Ea/RT} \qquad (3)$$

where E_a is the activation energy, A is the frequency factor, R = 8.314 J/(mol K) which is the ideal gas constant. The activation energy estimated from the inactivation data of *E. coli* was 10.79 kJ/mol and the frequency factor A, was 1.06 x 10^2 L/mg min. In general, the inactivation rate constants increased with an increase in temperature, but the values of the rate constants were less affected by temperature changes as compared to the effects observed for concentration.

Table 3.1. The Effect of temperature on the kinetics of chlorine dioxide (1.0 mg/L) inactivation of *E. coli* at pH 8.05

Organism	Temp(°C)	k $(min)^{-1}$	E_a (kJ/mol)	A (L/mg min)
E. coli	4	1.0255	10.79	1.06 x 10^2
	15	1.0644		
	22	1.3891		
	30	1.5579		
	37	1.5604		

3.3.3 Effect of pH.

Survival curves for the effect of changes of pH on the inactivation of ClO_2 on *E. coli* are as shown in Figure 3. 2. The inactivation efficiency was found to be significantly ($p < 0.05$) higher in the alkaline pH conditions when compared to pH of 6.5. When 1.0 mg/L was applied, less than 1 log (90%) of *E. coli* was inactivated at pH 6.5, whilst 4.0 log (99.99%) was achieved at pH 8.0 at the same reaction conditions. A comparison of the initial bacteria densities across the pH range used in this study showed no dependence of the different levels of inactivation on the pH of the solutions, suggesting therefore that the observed results emanate from the chemical nature of the disinfectant. ClO_2 is a strong oxidant that oxidises by its unique single electron abstraction where about 50 –70% of the initial dose reacts to produce chlorite (eq. (4)) and the redox potential of this reaction increases linearly with pH[36].

$$ClO_2 + e^- \rightarrow ClO_2^- \qquad (4)$$

Moreover, the disproportionation reaction of ClO_2 into chlorite and chlorate has been reported to be enhanced in a basic solution (eq.(5)) when compared to a slightly acidic or neutral solution[37].

$$2ClO_2 + 2OH^- \rightarrow ClO_2^- + ClO_3^- + H_2O \qquad (5)$$

This implies that the rate of abstraction of an electron from the organic components of the bacteria cell to cause inactivation is likely to be significantly faster in an alkaline medium relative to slightly acidic or neutral solutions. The available literature regarding the influence of pH on the biocidal efficiency of ClO_2 in water treatment has been contradictory. In general, the efficiency of ClO_2 as a disinfectant is often considered as being less impacted by variations of pH conditions in the range often encountered in water treatments,[10, 14] however, other findings have suggested strong pH sensitivity to ClO_2[18, 38]. The results of our present work suggest a strong pH dependency of chlorine dioxide in its bactericidal activity and this is of major significance in optimizing the disinfection process at water treatment facilities in practice.

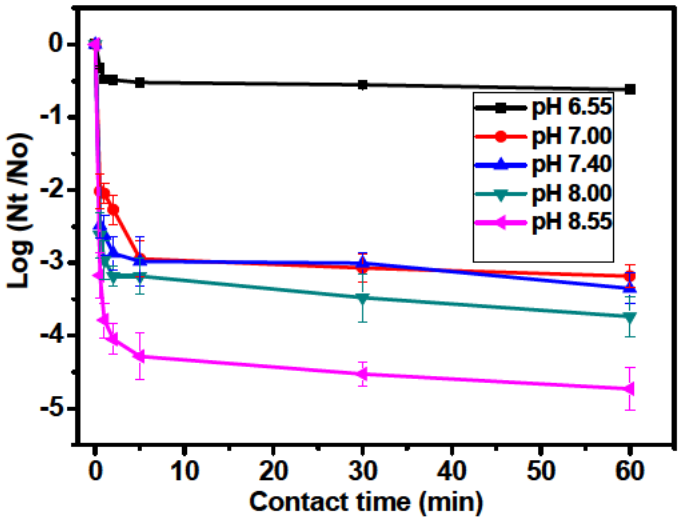

Figure 3.2. Effect of pH on the inactivation kinetics of *E. coli* exposed to 2.0 mg/L ClO_2 at 22 ± 2 ºC

3.3.4 The effect of microbial density on ClO_2 disinfection.

A plot of the survival ratios of ClO_2 inactivation of *E. coli* at different initial microbial densities (N_0) is shown in Figure 3.3. According to the experimental data, the susceptibility of *E. coli* to the disinfectant was found to be independent of the initial microbial density, but rather as a function of the concentration of ClO_2 applied. The analysis of the rate constants showed no significant difference ($p > 0.05$) in the rate of disinfection of ClO_2 at the different initial concentrations. Previous studies have reported of the significantly greater rate of inactivation at higher cell densities when *E. coli*, grown to the stationary phase was exposed to monochloramine[39]. Similar findings have also been observed of cell density-dependent acid resistance of *E. coli* cells at the stationary phase[40]. However, in both studies, the sensitivities of the cells in the exponential phase were found to be independent of the initial densities. The deviation of our results might be due to the differences in the oxidative strength of the different disinfectants employed. Monochloramine is relatively a weaker oxidant to ClO_2 and their fundamental reaction mechanisms also differ.

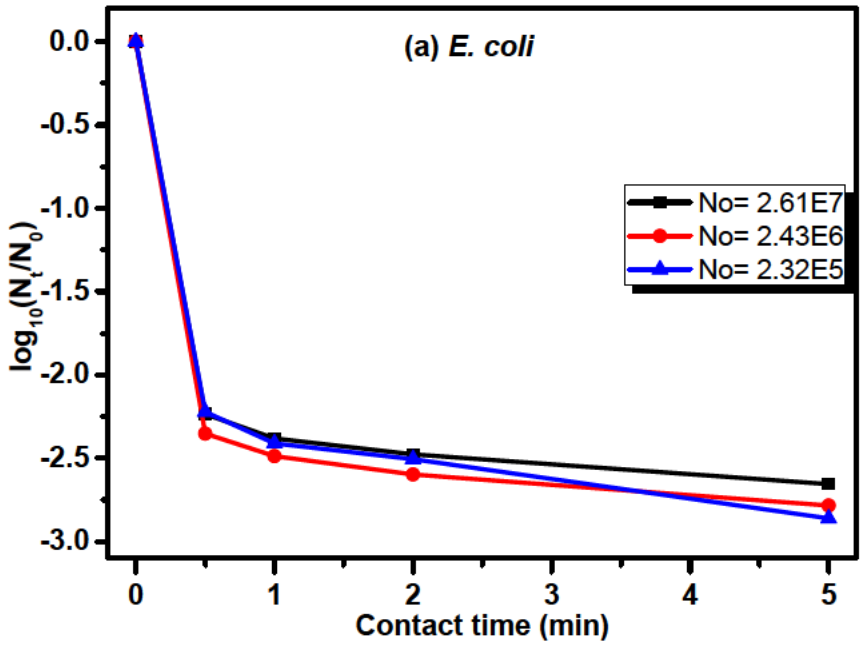

Figure 3.3 Effect of initial cell density on the ClO_2 (0.75 mg/L) inactivation kinetics of *E. coli*

3.3.5 Mechanism of action of chlorine dioxide

3.3.5.1 Effect of ClO_2 on the outer cell membrane.

The results of the NPN uptake assay of *E. coli* upon exposure to ClO_2 are shown in Table 3.2. Results are expressed as either relative fluorescence units (fluorescence value of cell suspension with NPN and without ClO_2 subtracted from the corresponding value of cell suspension and NPN with chlorine dioxide) or alternatively as NPN uptake factors. The uptake factors represent the ratio of the relative fluorescence units (background corrected fluorescence units) to that of the buffer[30]. Exposure of the cell suspensions to ClO_2 resulted in a significant increase in the NPN uptake by *E. coli* with a corresponding increase in the concentration of ClO_2. An intact Gram-negative bacterial outer cell membrane acts as a selective permeability barrier to hydrophobic macromolecules such as NPN as well as large hydrophilic molecules, [41] but it allows small hydrophilic molecules like nutrients to diffuse through the water-filled porin channels into the cell.

Table 3.2. Effect of chlorine dioxide on 1-*N*-phenylnapthalene (NPN) uptake by *E. coli*

Sample	NPN	Fluorescence (Mean+SD)	Background Subtracted Fluorescence	NPN Uptake factor
HEPES Buffer (5 mM, pH 7.2)		72.3 ±2.4		
Cell suspension only	+	242.0 ±2.9	169.7	2.34
Cells + ClO_2(1.0 mg/L)	_	255.3 ± 2.0		
Cells + ClO_2 (1.0 mg/L)	+	467.3 ± 13.0	395.0	2.93
Cells + ClO_2 (2.5 mg/L)	_	283.9 ± 2.0		
Cells + ClO_2 (2.5 mg/L)	+	643.0 ± 7.3	570.7	4.96

The NPN is a non-polar hydrophobic molecule and when it finds entry into the glycerophospholipid environment of the outer membrane, fluoresces strongly to produce a characteristic bright emission peak indicating a permeabilized or defective outer membrane[29]. This damage also leads to the leakage of some periplasmic constituents including proteins and oligosaccharides into the cell's environment. The observed increases in fluorescence due to the

exposure of the cell's suspension to ClO_2, therefore, suggest that an alteration of the integrity of the outer membrane had occurred.

The outer membrane permeability is vital to the activity of a relatively non-specific antimicrobial oxidant such as ClO_2 which may usually oxidise multi-target sites within the microbial cells but is essentially required to traverse the outer layers to reach their inner targets. Moreover, at the doses of ClO_2 applied (1.0 and 2.5 mg/L) in this case, the corresponding response between bacterial survival (Figure 1) and the NPN uptake also indicates the significance of the outer membrane permeability alteration to the biocidal activity. For instance, *P. aeruginosa* which is an opportunistic pathogen in most hospital environments has been demonstrated to be resistant to a wide range of antibiotics as a result of poor permeability to its outer membrane[42]. However, contrary to this, in other related studies [43-44], the bactericidal effects of hypochlorous acid and hypochlorite were found not to be linked to the changes in the membrane integrity of *E. coli* and therefore an increase in the outer membrane permeability alone may not necessarily explain the primary lethal event. Nevertheless, the outer membrane permeability is vital to the overall transport process leading to the bactericidal activity of the oxidant.

3.3.5.2 Effect of ClO_2 on the bacterial cytoplasmic membrane.

Figure 3.4 summarises the results obtained from the ONPG hydrolysis reaction by the intracellular β-D-galactosidase enzyme in *E. coli.* The treatment of the cells by ClO_2 at concentrations of 0.5 and 1.0 mg/L both resulted in almost a complete masking of the hydrolysis reaction with no yellowish *o*-nitrophenol (ONP) formed. ClO_2 is known to rapidly oxidise phenols in solutions and therefore to prevent interferences by such reactions it was effectively quenched by sodium thiosulphate before initiating the ONPG hydrolysis reaction. Similarly, equal amounts of the quenching agent were also applied in the control experiments of chloroform and untreated cells, which did not appear to produce any limiting effect on the enzyme activity. The observation, therefore, indicates the penetration of the ClO_2 molecules into the cytoplasmic membrane and subsequently causing the degradation and inactivation of the intracellular β-D-galactosidase enzyme. This eventually leads to inhibition of the hydrolysis reaction.

Chlorine dioxide has been found to denature critical constituents of proteins by oxidatively modifying the tyrosine and tryptophan residues and that has been suggested as the cause of inactivation in microorganism[45]. A rapid oxidation of the sulfhydryl (-SH) groups in cysteine by ClO_2 to form the disulfide (-S-S-) bridges in cystine resulting in the loss of activity of intracellular glucose oxidase activity and microbial death has also been reported[14, 46]. The reaction of ClO_2 was found to rapidly degrade β-D- galactosidase at levels beyond 0.6 log inactivation,[24] completely inhibit total dehydrogenase enzymes in bacteria within the first 5s of exposure even in the presence of a considerable population of bacteria remaining viable[23]. However, those findings could not link the loss of activity to the primary lethal lesion. A comparison of the results of bacteria inactivation at 0.5 mg/L (Figure 3.1) with the rapid and total inhibition of the enzymatic activity seeks to suggest that, the primary lethal effect of ClO_2 does not involve the disruption of the bacterial enzymatic activity. However, it provides further evidence of its penetration and interaction on the cytoplasmic membrane.

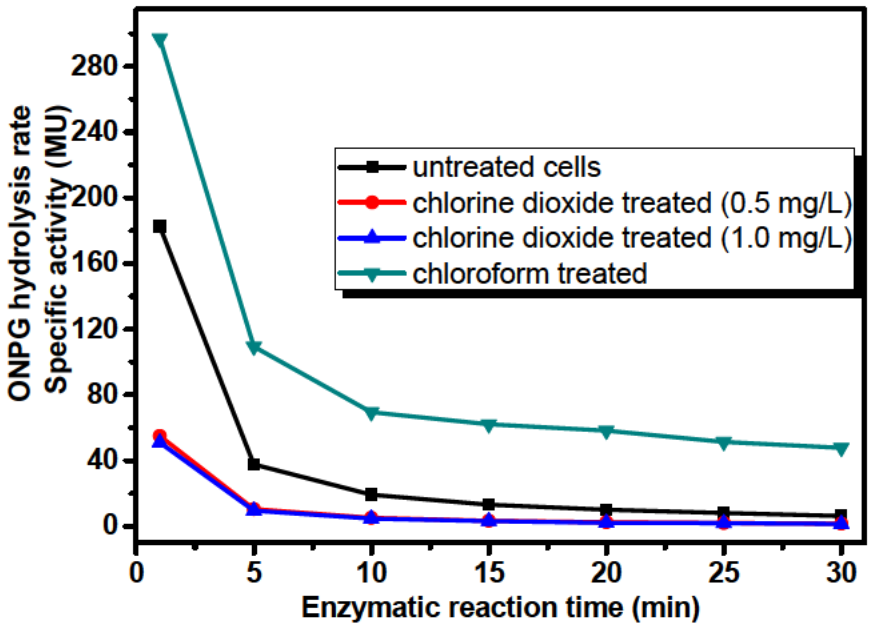

Figure 3. 4 Effect of ClO_2 on the enzymatic activity of β –D- galactosidase on ONPG

To further ascertain the disruption of the cytoplasmic membrane, an assay which involves the leakage of the 260 nm absorbing materials was also conducted. When the integrity of the

cytoplasmic membrane is compromised during a biocidal stress, it may result in the leakage of intracellular components such as nucleic acids. These components absorb UV strongly at 260 nm and are described as '260 nm absorbing materials[47]. The release of these cytoplasmic constituents into the extracellular environment (Supporting Information, Figure S3.2, page 69) was therefore monitored and related to the disruption of the cytoplasmic membrane. The degree of leakage was expressed as a ratio of the absorbance of the ClO_2 treated cells to the baseline untreated cells to account for differences in absorbance that might be arising out of different cell densities in the assay tubes. The curves generally showed linearity within the initial 5 min, after which a tailing off occurred. The relatively shorter period required for reaching maximum levels of the leakage at a given concentration could also be observed in the inactivation survival curves (Figure 3.1) of the bacteria cells. In control experiments, the samples of ClO_2 spiked into a phosphate buffer but without the bacterial cells did not absorb at all at 260 nm. Therefore, the observed increase in absorbance with concentration could only be attributed to the resulting oxidative damage caused by the ClO_2 molecules at higher concentrations. The integrity of the cytoplasmic membrane is maintained by a combination of non-covalent interactions between ionic, hydrophobic and hydrogen bonding,[48] and this is vital for the normal functioning of the bacterial cell. An alteration caused by an intrusion of the molecules of an oxidant such as ClO_2 tends to disrupt the balance of this interaction and thereby leading to the extensive loss of critical molecules and ions out from the membrane contents and eventual cell death[49].

3.3.5.3 Morphological changes by TEM.

The electron micrographs in Figure 3.5 compare the TEM ultrastructure images of ClO_2 treated and untreated cells of *E. coli*. In general, ClO_2 was not found to induce observable gross morphological alterations to the outer and inner membranes of *E. coli*. An analysis of about twelve *E. coli* micrographs showed that there was no significant difference ($p > 0.05$) between the mean cell wall thicknesses before treatment (26.83 nm) and after treatment (26.07 nm). ClO_2 is a small monomeric non-ionic gaseous molecule with high solubility in water. These properties may facilitate its diffusion into the bacteria resulting in increased permeability of the outer membrane and cytoplasmic membrane without necessarily lysing the cell.

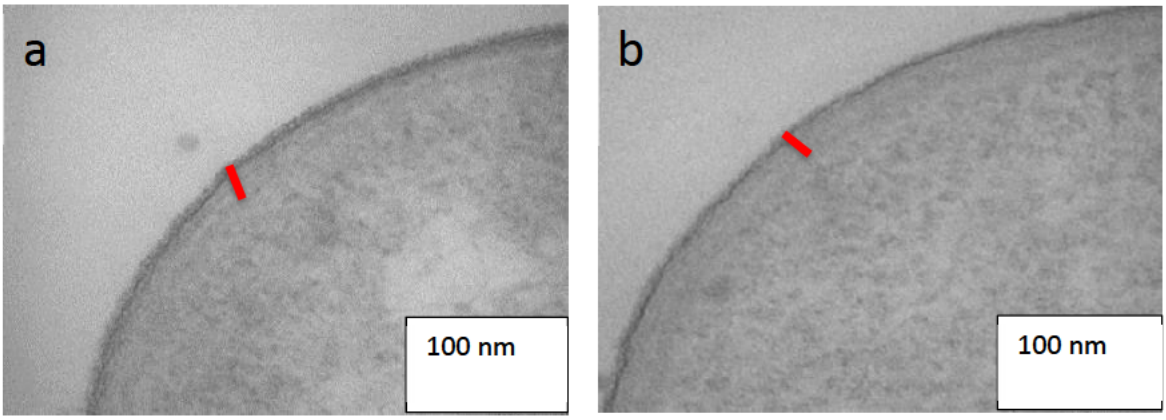

Figure 3.5 TEM images of bacteria before and after exposure to 2.0 mg/L chlorine dioxide (22 ± 2 °C, pH 8.0) (a) *E. coli* before treatment (b) *E. coli* after treatment

3.4 Conclusion

The results of this study have demonstrated that ClO_2 is a very fast and effective disinfectant against *E. coli* in water. However, its bactericidal activity is significantly impaired by dilution up to 0.5 mg/L. In designing an effective disinfection criterion in a contactor system with chlorine dioxide for potable or wastewater treatments, the principal consideration must be the application of adequate concentrations to achieve a desired level of inactivation rather than the application of lower dosages at longer contact times. The use of contact times to obtain a desired level of inactivation is practically inefficient with ClO_2. Moreover, the bactericidal activity of ClO_2 is significantly enhanced in an alkaline pH as compared to a neutral or slightly acidic medium. Higher dosages of ClO_2 may be required to be applied to water of acidic pH to achieve a comparable level of microbial inactivation as an alkaline water. ClO_2 inhibits the enzymatic activities of Gram-negative bacteria but that may not necessarily be responsible for the primary lethal effect. ClO_2 does not cause gross morphological damage to the Gram-negative cell wall but instead increases the permeability of the outer cell membrane, disrupting the integrity of the inner cytoplasmic membrane which leads to the efflux of intracellular contents of the cell and that may eventually result in the overall cell death. Future research on the potential development of resistant strains of bacteria due to the exposure to sub-optimal dosages of ClO_2 during water disinfection is envisioned.

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Supporting Information

Chlorine dioxide oxidation of *Escherichia coli* in water- A study of the disinfection kinetics and mechanism

Text S3.1

Preparation of bacterial suspensions for ClO_2 disinfection

Bacterial cultures were prepared for disinfection experiments by transferring stock cultures into 100 mL of Lysogeny broth (LB) (Merck, South Africa) in a 250 mL Erlenmeyer flask and incubated for 18 - 24 h at 37 ºC. Bacterial cells were then harvested by centrifuging at 6000 *x* g for 5 min at 4 ºC and washed twice in phosphate buffered saline (PBS, 10 mM at pH 7.4) and pellets suspended in an oxidant demand free (ODF) buffered water at an initial density of approximately 10^8 cfu/mL.

The sampled influent wastewater was filtered with a Whatman Number 1 filter paper (11µm) to remove suspended solid particles and 250 mL portions put into the reactors for the disinfection procedures as described in the experimental section.

Text S3.2

The release of β-D- galactosidase assay

After exposure to chlorine dioxide concentrations of 0.5 and 1.0 mg/L for 10 min the disinfection reaction was quenched with a 0.1 M sodium thiosulphate solution to prevent interferences resulting from ONP oxidation by chlorine dioxide. An aliquot of 4.0 mL of the disinfected cell suspension was mixed with 1.0 mL of 5 mM ONPG (Sigma Aldrich, USA) and incubated at 28 ºC in a water bath. A 0.1 mL of 1.0 M sodium carbonate/bicarbonate buffer (pH 10.0) was added to 0.9 mL aliquot of the reaction matrix in a cuvette (10 mm) at 5 min intervals for 30 min to both stop the reaction between the intracellular enzyme and the ONPG and to maximize absorbance. The rate of ONPG hydrolysis was determined spectrophotometrically (Shimadzu UV-3600, UV-VIS-NIR) by measuring changes in absorbance at 420 nm. The 0% absorbance for the spectrophotometer was set with the washed

cells in the buffer without ONPG to cater for the turbidity due to the whole cells. Additional experiments were conducted using chloroform to determine the maximum β-D-galactosidase activity. In this activity, a 0.25 mL of chloroform was added to similar aliquots of the washed cell suspensions like chlorine dioxide in 15 mL centrifuge tubes and after mixing for 10 min, the ONPG hydrolysis assay was carried out as above. The hydrolysis of the intact whole cells without chlorine dioxide treatment was similarly carried out as a control as above.

Text S3.3

Transmission electron microscopy (TEM) imaging

Suspensions of *E. coli* were treated with 2.0 mg/L ClO_2 for 10 min in a batch reactor. Approximately 1.0 mL of the treated samples were pipetted into Eppendorf tubes and pelleted by centrifuging at 5000 *x g* for 5 min at 25 ºC. Pelleted samples were fixed with a phosphate buffered (pH 7.4) 2.5% glutaraldehyde solution and kept overnight at 4 ºC. The sample suspension was washed in 10 mM phosphate buffer (pH 7.4) and suspended in 0.5% osmium tetroxide for post-fixation. Dehydration of samples was gradually done with 30%, 50%, 75% and 100% acetone and infiltrated first with equal parts of resin and acetone for 4 h followed by whole resin for 20 h. The specimens were orientated in a mold of whole resin and polymerized in an oven for 8 h at 70 ºC. Thin sections of samples were prepared with Ultra-Microtome (LEICA EM UC7, Germany) and stained with 2% uranyl acetate and subsequently with lead citrate. The prepared grids were examined using JEM -2100 (JEOL Co. Japan) and the changes in the cell wall thickness were analysed with iTEM E - 23082007 software (Olympus Soft Imaging Solutions GmbH, Germany)

Table S3.1. Kinetic parameters of chlorine dioxide inactivation of *E. coli* at various concentrations fitted to the C_aH model

$[ClO_2]$ / mgL^{-1}	*k*	*m*	*n*
0.5	0.3831	0.2015	0.34
1.0	2.2406	0.1172	0.6956
2.0	2.3897	0.0543	0.5819
3.5	2.3896	0.0031	0.5507
5.0	2.4247	0.0001	0.4587

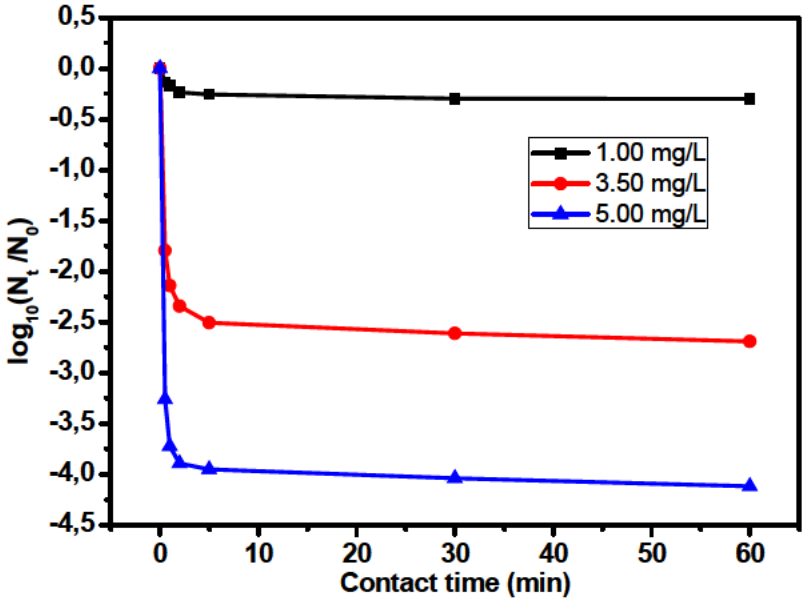

Figure S3.1. Inactivation survival curves of bacteria population (2×10^5 cfu/mL) in a waste water sample (COD; 101.7 ± 5.9 mg/L, pH 7.2 at 22 ± 2 ºC) exposed to different ClO_2 dosage.

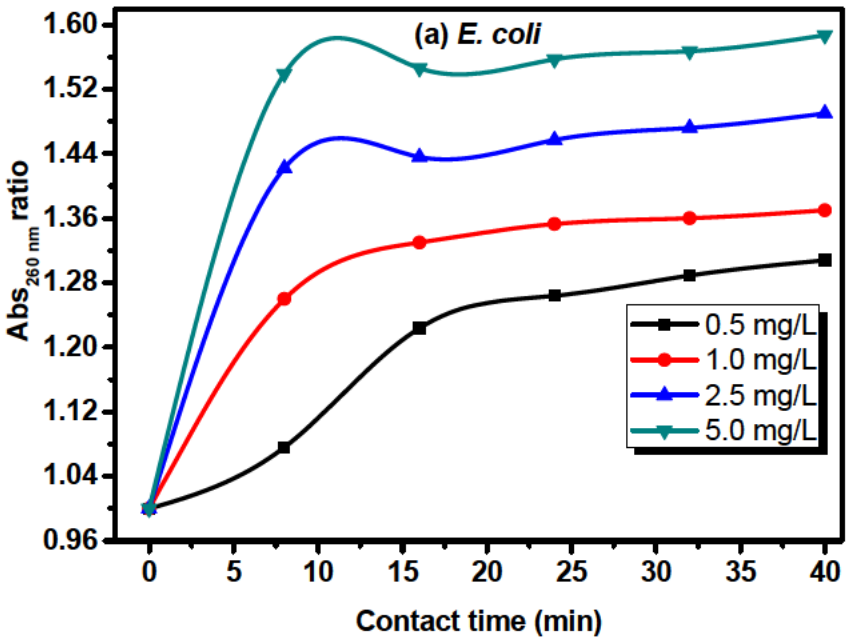

Figure S3.2. Absorbance profile of 260 nm absorbing materials of *E. coli.* exposed to different concentrations of ClO_2.

CHAPTER 4

Inactivation Kinetics and Mechanism of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in water: The case of Chlorine Dioxide

Abstract

This study evaluated the inactivation kinetics and the bactericidal mechanism of chlorine dioxide towards *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 29213) on a laboratory scale with the view of determining the optimal operational conditions of its application as a disinfectant in water. Bacteria inactivation was conducted in batch reactors at varied disinfectant concentrations, pH, temperature and initial bacteria densities in buffered disinfectant demand free water. The bactericidal mechanism in terms of the effect on the permeability of the outer and cytoplasmic cell membranes and the morphology of the cells were monitored. At the highest studied concentration (5.0 mg/L), at least 5-log reductions in bacterial population were observed for each strain of bacteria. Chlorine dioxide inactivation showed a stronger sensitivity to changes in water pH conditions with the inactivation rate at 8.5 being at least 4-fold of what pertained at 6.5 but efficiency was less impacted by changes in the initial bacteria density. A rise in temperature from 4 ºC to 15 ºC resulted in approximately 56% increase in the inactivation rate of *S. aureus*. Chlorine dioxide was found to increase the permeability of outer and cytoplasmic cell membranes and consequently resulting in the release of vital nuclear materials which strongly correlated with loss of cell activity or death. However, from TEM micrographs significant morphological damages or cells lysis was not observed. These results provide vital data on operational strategies to enhance efficient disinfection of water with chlorine dioxide.

KEYWORDS: Chlorine dioxide, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, Disinfection, Kinetics

4.1 Introduction

Chlorine dioxide (ClO_2) is a powerful oxidant with broad-spectrum biocidal activity[1]. It has thus found considerable application as a sanitiser in the food industry[2-3], decreasing biofouling in industrial cooling water systems[4-5] , disinfection of potable and wastewater[6-7] and in the pulp industry as a bleaching agent for producing excellent strong and bright fibers[1, 8]. Most especially, in potable water and wastewater disinfection, it has proven to be an excellent alternative technology to conventional chlorination against the threats of potential waterborne pathogenic and infectious agents such as bacteria, viruses, and protozoa[9-11]. Moreover, besides the regular application as a disinfectant in water treatment, recent reports have also indicated promising potentials for the oxidation and removal of pharmaceutical residues such as diclofenac[12], tetracyclines[13] and sulfamethoxazole[14] in wastewaters.

Chlorine dioxide inactivates microbial threats by selective oxidation where a single electron is usually abstracted. This results in the formation of chlorite (ClO_2^-) and chlorate (ClO_3^-) as the main by-products with chlorite constituting approximately 70 %[15] of the total reaction by-products.

$$ClO_2 + e^- \rightarrow ClO_2^- \quad (1)$$

$$2ClO_2 + 2OH^- \rightarrow ClO_2^- + ClO_3^- + H_2O \quad (2)$$

Chlorites and chlorates levels in drinking water are increasingly being regulated due to potential health concerns associated with them. The WHO recently reaffirmed a maximum acceptable threshold for chlorite in drinking water as 0.7 mg/L[16]. The US EPA also regulates the maximum contaminant level (MCL) for chlorite in drinking water at 1.0 mg/L[17]. Consequently, the maximum residual disinfectant levels (MRDL) for chlorine dioxide in drinking water is enforced at 0.8 mg/L in the United States. The associated challenge in the potable water treatment industry thus lies with satisfying the regulatory guidelines without compromising the adequacy of disinfection. This is further exacerbated by the increasing reports of some bacteria strains developing resistance to antibiotics in water due to inadequate disinfection[18-19].

In this regard, designing and operating an efficient chlorine dioxide disinfection system for either potable water or wastewater and simultaneously controlling the formation of deleterious disinfection by-products would require due consideration of suitable operating conditions to a

target microorganism. In a recent communication[20], we assessed the efficiency of chlorine dioxide as a disinfectant against *Escherichia coli* under different operational conditions of water treatment in addition to a proposed bactericidal mechanism. However, it is well established that the intrinsic susceptibilities of organisms to disinfectants vary widely[21-22] and thus an appropriate determination of optimal conditions for a disinfectant would partly be dependent on the target organism. A broader data on the perspective of chlorine dioxide disinfection of other bacterial species becomes necessary.

Pseudomonas aeruginosa is a Gram-negative bacterium that is ubiquitous in the environment and able to adapt to several conditions. It is often described as an opportunistic pathogen due to its association with life-threatening ailments in burn and surgical patients and in immunocompromised patients in hospital environments. It is also a common cause of infections in patients suffering from cystic fibrosis[23]. *P. aeruginosa* could also be found in a water including swimming pools and tap water[24-25] and indeed the transmission through hospital tap water route has been described as highly significant[26]. *Staphylococcus aureus*, on the other hand, is prevalent in diverse food products such as meat and milk[27-28]. The methicillin-resistant *S. aureus* (MRSA) strains have particularly gained considerable attention in recent times owing to the risks of transmitting potential infections from animals to humans[29]. In previous efforts at controlling these strains of bacteria with chlorine dioxide, relatively high concentrations were employed[30-31]. Such concentrations, however, may not meet regulatory requirements when applied for disinfecting drinking water. Moreover, a comprehensive data from a systematic study of chlorine dioxide inactivation of *P. aeruginosa* and *S. aureus* is quite limited.

In the present work, the kinetics of chlorine dioxide inactivation of *P. aeruginosa* and *S. aureus* was studied under varied conditions of oxidant concentration, water pH, temperature and initial bacterial density. The bactericidal mechanism of chlorine dioxide on the bacteria strains was also investigated.

4.2 Materials and methods

4.2.1 Bacterial strains and preparation of suspensions

Culture collection strains of *P. aeruginosa* (ATCC 27853) and *S. aureus* (ATCC 29213) were obtained from the Microbiology Discipline, University of KwaZulu-Natal, Westville. Bacteria cell suspensions were prepared for the inactivation studies by growing the stock cultures in 100 mL of Lysogeny broth (LB) (Merck, South Africa) contained in a 250 mL Erlenmeyer flask and incubated for 18 - 24 h at 37 ºC. Cells were then harvested by centrifuging at 6000 *x g* for 5 min at 4 ºC, washed twice in phosphate buffered saline (PBS, 10 mM at pH 7.4) and pellets suspended in an oxidant demand free (ODF) buffered water to an initial density of approximately 10^8 cfu/mL[20].

4.2.2 Chlorine dioxide preparation and measurement

Stock solutions of chlorine dioxide were prepared as described previously[20, 32] by oxidising approximately 25% (w/v) solutions of sodium chlorite ($NaClO_2$) in a gas generating bottle with a dilute solution of sulphuric acid (H_2SO_4, 2M). The generated gas was harvested through a stream of compressed air into a connecting chlorine scrubber system which contained a saturated solution of sodium chlorite (10 % w/v) to scrub contaminants such as chlorine gas. Chlorine dioxide gas was collected in a connecting bottle of demand free deionized water. The concentrations of the prepared stock solutions were analysed by the Iodometric method whilst the residual concentrations were determined by the N, N-diethyl-p-phenylenediamine (DPD) method [32].

4.2.3 Bacteria inactivation kinetics

Each of the inactivation kinetic reactions of *P. aeruginosa* and *S. aureus* with chlorine dioxide were carried out in a 500 mL of sterile oxidant demand free (ODF) deionized water buffered with phosphate buffered saline (PBS) in a glass batch reactor. Prior to each inactivation reactions, glassware were soaked in 10 mg/L chlorine dioxide solution for 4 h and thoroughly rinsed with deionized water to remove all possible chlorine dioxide consuming species that might interfere with the applied dose during the reaction. Bacterial suspensions estimated to yield a final concentration of ~ 10^8 cfu/mL based on prior calibrations were added to the water in the reactor and allowed to equilibrate with the surrounding temperature under continuous stirring with a magnetic stirrer to ensure uniform mixing throughout the reaction period. Samples of bacterial suspensions from the reactor were withdrawn before the addition of the chlorine dioxide to determine the initial bacteria populations. Subsequently, calculated desired

concentrations of chlorine dioxide (0.5, 1.0, 2.5, 3.5 and 5.0 mg/L) were added to initiate the reaction in the reactor. Five milliliters samples were withdrawn with 10 mL sterile syringes at predetermined time points into tubes containing excess 0.1 M sodium thiosulphate ($Na_2S_2O_3$) solutions to immediately quench the residual chlorine dioxide and consequently stopping the reaction. Enumerations of bacteria were determined by the standard spread plate technique on nutrient agar (Merck, South Africa) after appropriate dilutions and incubation at 37ºC for 24 h.

The influence of water temperature and pH on the rate and degree of chlorine dioxide inactivation of each bacterial strain considered in this study were also assessed. Inactivation kinetic reactions as described above were conducted at different temperatures with the reaction water matrix at pH 8.05 maintained at 4, 15, 22, 30 and 37 ± 2 °C and allowed to equilibrate with the surrounding temperature before initiating the reaction. Chlorine dioxide was applied at an initial dose of 2.0 mg/L in each case based on preliminary trials at room temperature which yielded at least 2-log (99%) inactivation credits of each bacteria. Such levels of reduction were considered appropriate to indicate possible significant effects of the operational parameters on inactivation. To determine the effect of changes in water pH on the inactivation kinetics, experiments were similarly carried out at 22 ± 2 ºC by varying the pH in the range of 6.5 – 9, considering the pH conditions usually encountered in natural water. It should be noted that all inactivation experiments for each bacterium were carried out independently and measurements made in triplicates.

4.2.4 Effect of bacterial density on the kinetics of ClO_2 disinfection

The bacteria cultures of the studied organisms were grown for 18 - 24 h into the stationary phase in a Lysogeny broth under agitation at 37 ºC. Cells were then harvested by centrifugation at 6000 x *g*, washed twice with sterile PBS (PH 8.05) and subsequently resuspended in a buffered 100 mL ODF water to obtain the stock bacteria density at an approximate count of 1-2 $x10^8$ cfu/mL. This was estimated from a predetermined data between optical densities (OD) measured at 600 nm with a spectrophotometer and cell density (cfu/mL). The stock cultures were serially diluted to obtain approximate cell densities of 10^7, 10^6 and 10^5 cfu/mL in 100 mL of the reaction matrix contained in 250 mL Erlenmeyer flasks. Each of the different densities of *S. aureus* and *P. aeruginosa* were treated with 2.5 mg/L and 1.0 mg/L of ClO_2 respectively which were expected to produce at least 2.0 –log inactivation as obtained from the experimental results of Section 4.2.3. Portions of each sample were withdrawn prior to chlorine dioxide treatment as a control and processed simultaneously to determine and confirm the initial

bacterial density in each case. Sampling and enumeration of bacteria were carried out as described above.

4.2.5 Mechanism of action of chlorine dioxide disinfection of bacteria

Experiments were designed to monitor the bactericidal mechanisms of chlorine dioxide in terms of the effect on the permeability of the outer cell membrane, the cytoplasmic membrane and the morphology of the cells.

4.2.5.1 Cell outer membrane permeability.

The non-polar permeability probe 1-N-phenylnaphthylamine (NPN) uptake assay as described previously [33-34] was used to determine the effect of ClO_2 on the permeability of the outer cell membrane of Gram-negative bacteria; *P. aeruginosa*. In brief, a 10 mM stock solution of NPN was prepared in acetone. A mid-logarithmic phase (OD_{600} value of 0.5 ± 0.02) bacterial cultures grown in 100 mL LB at 37 ºC were harvested by centrifugation at 6000 *x g* for 10 min, washed twice in HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) buffer (5 mM, pH 7.4) and then suspended in 15 mL fresh portions of the same buffer to obtain a cell stock. Approximately 3.0 mL each of the stock samples were withdrawn into tubes and exposed to varying concentrations of ClO_2 (1.0, 2.5 and 5.0 mg/L) for 10 min and quenched effectively with sodium thiosulphate to prevent the residual ClO_2 from interfering with NPN fluorescence. Negative control cell samples were identically processed except for the addition of ClO_2 whilst the treatment with EDTA (1.0 mM), which is known to possess strong outer membrane permeability characteristic was utilised as a positive control. NPN was subsequently added up to 10 µM final concentration. The changes in fluorescence as a function of time were monitored with a Perkin Elmer LS 55 fluorescence spectrophotometer (slit width = 5.0 nm, excitation λ = 350 nm and emission λ = 420 nm) up to a maximum where no further increases could be observed. The maximum fluorescence values were expressed as % NPN uptake = $(F_{obs} - F_o / F_{100} - F_o) \times 100$ where F_{obs} is the observed NPN fluorescence at a given concentration of ClO_2, F_o is the background NPN fluorescence of bacterial cell suspension in buffer but without ClO_2, and F_{100} is the fluorescence of NPN upon treatment with 1.0 mM of EDTA. Triplicate measurements were made for each sample[35].

4.2.5.2 Disruption of the cytoplasmic membrane

The effect of ClO_2 on the integrity of the cytoplasmic membrane was studied by monitoring the release of cytoplasmic components such as nucleic acids which absorb UV-light strongly at 260 nm. Overnight cultures as described above were harvested by centrifugation at 5000 *x g* for 10 min and twice washed in autoclaved PBS (10 mM, pH 7.4). Pellets were resuspended in 10 mL portions of the buffer and treated with varying concentrations of ClO_2 (0.5, 1.0, 2.5 and 5.0 mg/L). Control samples were identically processed except for the ClO_2 treatment. Approximately 2.0 mL of the cell suspensions were sampled at 5 min intervals and centrifuged (6000 *x g*, 2 min) immediately to separate the bacterial cells from the supernatant. The optical densities of the supernatants were then determined at 260 nm with a Nanodrop 2000C spectrophotometer (Thermo Scientific). It is to be noted that in this protocol, ClO_2 was not quenched before centrifuging because the quenching agent (sodium thiosulphate) was observed to absorb at a very close wavelength (254 nm) as the nucleic acids which might interfere with the measured absorbances. Chlorine dioxide solution, however, was not found to absorb at this wavelength. Results were expressed as:

$$Abs\ 260nm\ ratio = \frac{Absorbance\ of\ chlorine\ dioxide\ treated\ cells}{Absorbance\ of\ baseline\ untreated\ cells}$$

to normalize the differences in absorbance that might be due to different cell densities in the assay tubes.

4.2.5.3 Transmission Electron Microscopy (TEM) analysis

The changes to the morphology of the bacterial cells due to the chlorine dioxide treatment was examined by TEM [36-37]. In this process, suspensions of bacteria in PBS (10 mM, pH 7.4) were treated with 4.0 mg/L ClO_2 sufficient for yielding at least 4-log inactivation for 10 min in a batch reactor. Approximately 1.0 mL of the treated samples were pipetted into Eppendorf tubes and pelleted by centrifuging at 5000 *x g* for 5 min at 25 °C. Pelleted samples were fixed with a phosphate buffered (pH 7.4) 2.5% glutaraldehyde solution and kept overnight at 4 °C. The sample suspension was washed in 10 mM phosphate buffer (pH 7.4) and suspended in 0.5% osmium tetroxide for post-fixation. Samples were dehydrated sequentially with 30%, 50%, 75% and 100% acetone and infiltrated first with equal parts of resin and acetone for 4 h followed by a whole resin for 20 h. The specimens were orientated in a mold of whole resin and polymerized in an oven for 8 h at 70 °C. Thin sections of samples were prepared with Ultra-Microtome (LEICA EM UC7, Germany) and stained with 2% uranyl acetate and subsequently

with lead citrate. The prepared grids were examined using JEM -2100 (JEOL Co. Japan) and the changes in the cell wall thickness were analysed with iTEM E - 23082007 software (Olympus Soft Imaging Solutions GmbH, Germany).

4.2.6 Data Analysis

Bacteria inactivation data were best fit by the C_{avg} Hom (C_aH) model [38-39] expressed as:

$$\log (N_t / N_o) = -kC^n_{avg} T^m \quad (3)$$

$$C_{avg} = \sqrt{C_o.C_f} \quad (4)$$

where **(N_t/N_o)** is the survival ratio of the microorganism under consideration after inactivation, *k* is the microbial inactivation rate constant, C_o and C_f are the initial and final disinfectant concentrations (mg/L) respectively and T is the contact time of exposing the disinfectant to the microorganism, m is a model parameter called the Hom exponent whilst n is the *coefficient of dilution*. Model parameters were determined by minimising the error sum of square (ESS) deviations between the observed and predicted log (N_t/N_o) of the inactivation data using Microsoft Excel Solver (Microsoft Corp, 2016). The student *t*-test based on *p*-value at 95% confidence level was used to evaluate the statistical significance of data from independent measurements.

4.3 Results and Discussion

4.3.1 Inactivation kinetics at different chlorine dioxide concentrations

The log inactivation levels for chlorine dioxide disinfection of *P. aeruginosa* and *S. aureus* are summarised in Figure 4.1. Expectedly, the inactivation credits obtained generally increased with an increase in the applied initial concentration of chlorine dioxide. The inactivation curves of the applied doses were typically nonlinear. Instead, they were characterised by a rapid initial exponential reduction of the bacterial population, followed by a slow kinetic phase to produce a tailing. Moreover, the corresponding levels of inactivation were predominantly a function of the applied dose when compared with the influence of the contact time. This was evident in the observation that at any applied concentration of chlorine dioxide, the differences in the level of log inactivation obtained at t = 2 min and t = 60 min were mostly statistically insignificant ($p > 0.05$). It thus suggests that the use of CT (disinfectant concentration, C x contact time, T) credits to estimate the efficiency of inactivation largely becomes unreliable.

In comparing the susceptibilities of the bacterial strains, the log inactivation level should be put into consideration. For instance, at 1.0 mg/L ClO_2 dose, ~ 2-log reduction of *P. aeruginosa* was observed whilst 2.5 mg/L dosage was rather required to achieve at least 2.0 log inactivation for *S. aureus*. On the contrary, when cells were exposed to 5.0 mg/L, approximately 5 and 6 log reductions for *P. aeruginosa* and *S. aureus* respectively were obtained. Several interpretations of this phenomenon have been proposed and chiefly among them is the vitalist hypothesis of chemical disinfection[40]. In this principle, it is suggested that a strain of bacteria consists of subpopulations of different fractions (dependent on the type of organism) which intrinsically possess diverse susceptibilities to a disinfectant exposed at given time[41]. From this assumption, it could be presumed that the more susceptible fractions of *P. aeruginosa* were rapidly inactivated at 1.0 mg/L whilst higher concentrations of chlorine dioxide were required to effectively kill the relatively more resistant subpopulations.

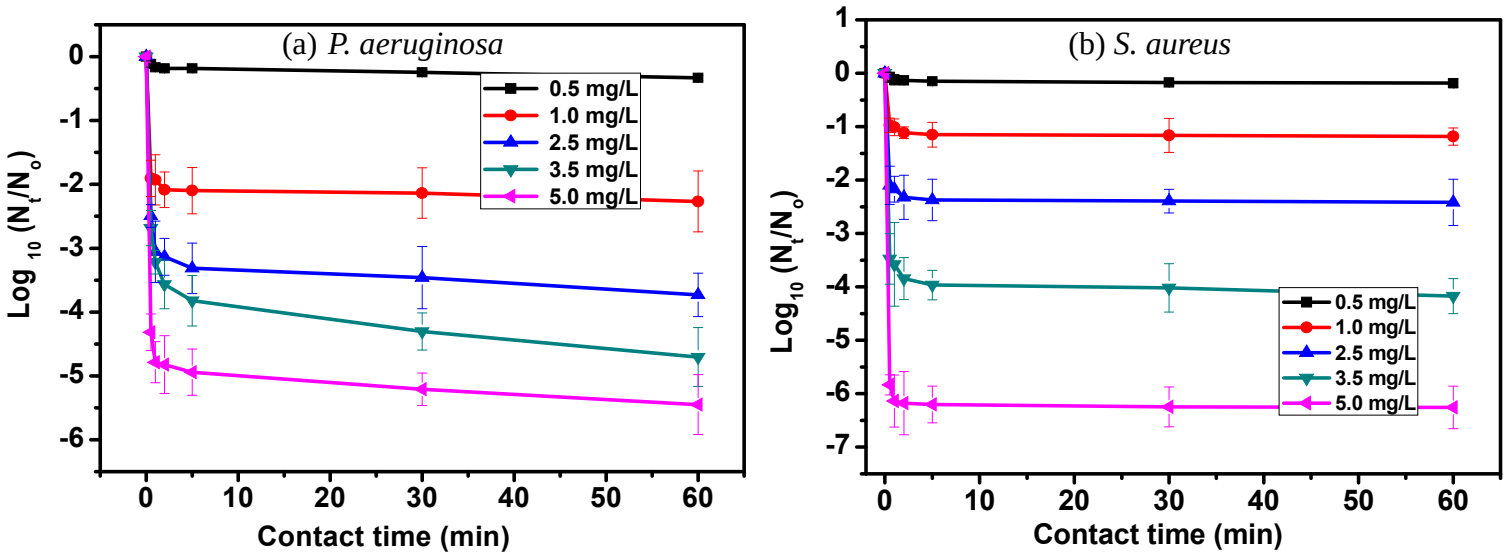

Figure 4.1. Kinetics of chlorine dioxide inactivation of *P. aeruginosa* and *S. aureus* at varied oxidant concentrations. Data determined at pH 8.05 and 22 ± 2 ºC

Similarly, relatively higher doses were required to produce a significant change in the population of *S. aureus* due to the presence of more resistant fractions. The declining rate of inactivation is thus a direct consequence of the diversity of the bacterial populations possessing different responses to the different concentrations of the disinfectant. However, notably, 0.5 mg/L was virtually inactive to the strains of bacteria considered in this study. For example, notwithstanding the amount of contact time allowed for a reaction within 1 h, less than 0.3 log inactivation was recorded. Usually, in drinking water, it is recommended that the applied dose should not exceed 1.5 mg/L[7]. On the basis of the results of our study, such concentrations may not be adequate for inactivating high levels of bacterial population in water.

In Table 4.1, a summary of the inactivation rate constants and the estimated model parameters from the C_{avg} Hom model (eq. (3)) is presented. These were computed by minimising the Error Sum of Squares (ESS) between the log (N_t/N_o) values from the observed experimental data and the predicted from the C_{avg} Hom using the Solver function in Microsoft Excel (version 2016). From the table, a value of $m < 1$ is an indication that the microbial survival plot at a given concentration shows a tailing-effect whereas a shoulder is signified by $m > 1$[39]. An increase in the rate of inactivation is indicated by a higher *k* value whilst *n* shows the sensitivity of the concentration to the rate of inactivation[38]. The model parameters further emphasise the non-linear characteristic of chlorine dioxide inactivation of *P. aeruginosa*, *S. aureus* as well as *E. coli* [20] in water

Table 4.1. Estimated parameters of fitted C_{avg} Hom model for chlorine dioxide inactivation of *P. aeruginosa* and *S. aureus* determined at pH 8.05 and 22 ± 2 ºC

Bacteria	**[ClO_2]/ mg/L**	**Parameter**			
		k	***m***	***n***	**R^2**
P. aeruginosa	0.5	0.407	0.165	1.261	0.995
	1.0	2.021	0.001	1.260	0.995
	2.5	1.974	0.227	0.503	0.993
	3.5	1.791	0.199	0.470	0.991
	5.0	2.378	0.243	0.492	0.971
S. aureus	0.5	0.251	0.232	1.230	0.982
	1.0	1.724	0.076	1.229	0.993
	2.5	0.908	0.055	0.960	0.995
	3.5	0.964	0.060	1.056	0.974
	5.0	0.994	0.001	1.132	0.995

4.3.2 Effect of pH on chlorine dioxide disinfection of bacteria.

The variation of water pH on the kinetics of bacterial inactivation by chlorine dioxide is shown in Figure 4.2. The plot depicts the inactivation rate constants of the kinetic reaction between chlorine dioxide and the bacterial cells at various water pH conditions where the model parameters *m* and *n* from Eq. 3 were assumed to be unity (n set as 1 because only a single concentration was utilised)[42]. It must be emphasised that for all inactivation experiments the initial densities of bacteria were not affected by any of the pH conditions considered in the present study. In determining the rate constants, only the rapid initial phase which followed the first-order kinetics was considered because the difference between the log inactivation data

at the transition points and the end of the tailing phase of the kinetic curves (data not shown) were largely found to be statistically insignificant ($p > 0.05$). The rate of bacterial inactivation essentially increased with increasing pH and the most effective inactivation measurements were recorded in the alkaline pH conditions. Under similar experimental conditions, varying the pH from 6.5 to 8.5 resulted in approximately 4-fold and 7- fold increases in the inactivation rates of *S. aureus* and *P. aeruginosa* respectively. These results profoundly indicate a substantial influence of water pH on the efficiency of chlorine dioxide disinfection. The role of pH has considerably become a subject of contradiction among researchers. Unlike free chlorine, it is widely regarded that the effectiveness of chlorine dioxide spans over a wide range of pH conditions[10, 43] and essentially unaffected by changes in water pH. Conversely, other researchers have reported significant influences of pH on the efficiency of chlorine dioxide inactivation of microbes[44-45].

It has been suggested that the observed increased reactivity of chlorine dioxide under alkaline conditions may be due to the formation of more reactive transient species consisting of the OH^- ions, chlorine dioxide, and the substrate[46]. The rapid reactions of chlorine dioxide with cysteine and glutathione under alkaline conditions, which are essential components of several enzymatic activities in bacterial cells[47] have also been suggested to be responsible for these observations. In our previous work[20], we indicated that the alkaline pH conditions accelerate the decay rate of chlorine dioxide[48-49] and the mechanism of the process enhance the rate of oxidation of microbial cells. At this stage, the chemistry explaining the significantly high inactivation efficiency in alkaline pH conditions is inconclusive. However, the implications in practice are that residual doses in drinking water distribution systems under high pH conditions are likely to decompose faster, thus exposing the water to recontamination in the distribution system[48]. Combining chlorine dioxide with more stable disinfectants such as monochloramine or free chlorine is thus desired[50].

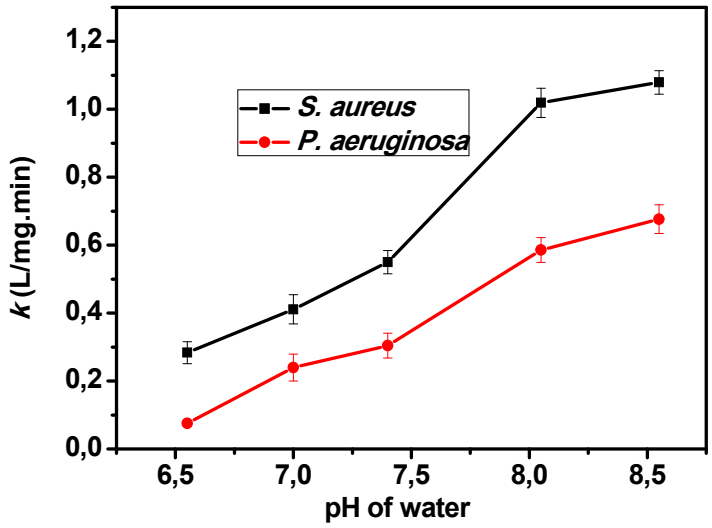

Figure 4.2. Influence of varying water pH on the inactivation rate constants of *P. aeruginosa* and *S. aureus* exposed to 2.0 mg/L chlorine dioxide at 22 ± 2 ºC

4.3.3 Effect of temperature.

Temperature is a vital physical parameter that impacts on the efficiency of chemical disinfectants. The effect of temperature on the pseudo-first order inactivation rate constants after exposing the bacteria strains to 2.0 mg/L chlorine dioxide is shown in Figure 4. 3. The rate constants were determined from the rapid initial phase or the log-linear region of the disinfection curves corresponding to a first-order kinetic[51]. A significant difference ($p < 0.05$) between the inactivation rate constants at the temperature range considered was observed for both *P. aeruginosa* and *S. aureus.* Particularly, for *S. aureus*, raising the temperature from 4 ºC to 15 ºC resulted in ~ 56% increase in the inactivation rate. Generally, the rate of inactivation increased steadily with increasing temperature from 15 ºC to 37 ºC. The dependence of the inactivation rates at the studied temperatures were further analysed by the Arrhenius equation

$$k = A\, e^{-\frac{Ea}{RT}} \qquad (5)$$

where k is the inactivation rate constant, E_a is the activation energy, A is the frequency factor, T is the absolute temperature in Kelvin and the universal gas constant R = 8.314 J/mol.K.

The Arrhenius parameters E_a and A estimated from equation 5 for *P. aeruginosa* were 7.73 kJ/ mol and 12.3 L/mg.min respectively. In the case of *S. aureus*, the E_a was 13.26 kJ/mol and A determined as 2.73 x10^2 L/mg.min. With chemical disinfection often considered as akin to chemical reactions[40], it is presumed that the enhanced activity of a disinfectant with

temperature is largely due to the increased effective collisions of the disinfectant's molecules with the bacterial cells which eventually initiate the oxidative processes leading to cell death.

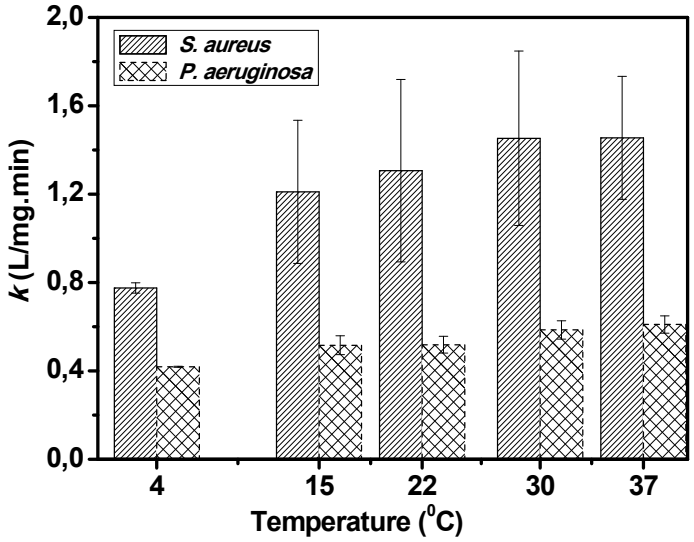

Figure 4.3. The variation of water temperature on the inactivation rate constants *k,* of *P. aeruginosa* and *S. aureus* exposed to 2.0 mg/L chlorine dioxide (pH 8.05)

4.4 Effect of Initial density of bacteria

The influence of the initial bacteria density on the efficiency of chlorine dioxide was also studied. Figure 4. 4 illustrates the inactivation kinetics of the bacterial strains exposed to concentrations sufficient to yield at least 2-log inactivation credits. In general, no significant difference ($p > 0.05$) between the inactivation rate constants was obtained for the different densities of bacteria in any of the studied organisms. A further comparison of the results herein with section 4. 3.2 where the initial cell density was maintained at ~ 10^8 cfu/mL tend to suggest that under similar experimental conditions, the rate of inactivation and for that matter the log inactivation credits obtained for a target organism exposed to chlorine dioxide would be less impacted by the initial bacteria density. In a similar study conducted in a batch reactor using monochloramine[52], it was shown that the initial bacteria density of *E. coli* grown to the stationary phase was a statistically significant parameter in determining the inactivation rate. However, the contrary was observed for cells in the exponential phase. Another related work[53] involving monochloramine and ozone applied to *E. coli* and *Bacillus subtilis* cells and spores

observed that the influence of the initial density was only related to the reaction of monochloramine with stationary phase cells of *E. coli*. It was thus concluded that the effect of initial density on disinfection is dependent on the growth phase and type of target organism. Quorum sensing in bacterial cells is a cell-cell communication mechanism employed by individual cells to assess the gene expression, diffusion gradient and local densities in a multicellular-like behaviour[54-55]. This phenomenon has been associated with the induction of resistance among high-density cells towards antibiotics and antimicrobials[56]. However, in our present work, the effect of this was not observed under the experimental conditions. Probably, chlorine dioxide as a strong oxidant target multiple sites[57-58] within the cell thus making it unlikely for cells to develop resistance by specific gene expression mechanisms or possibly the cells were exposed to relatively high disinfectant concentration.

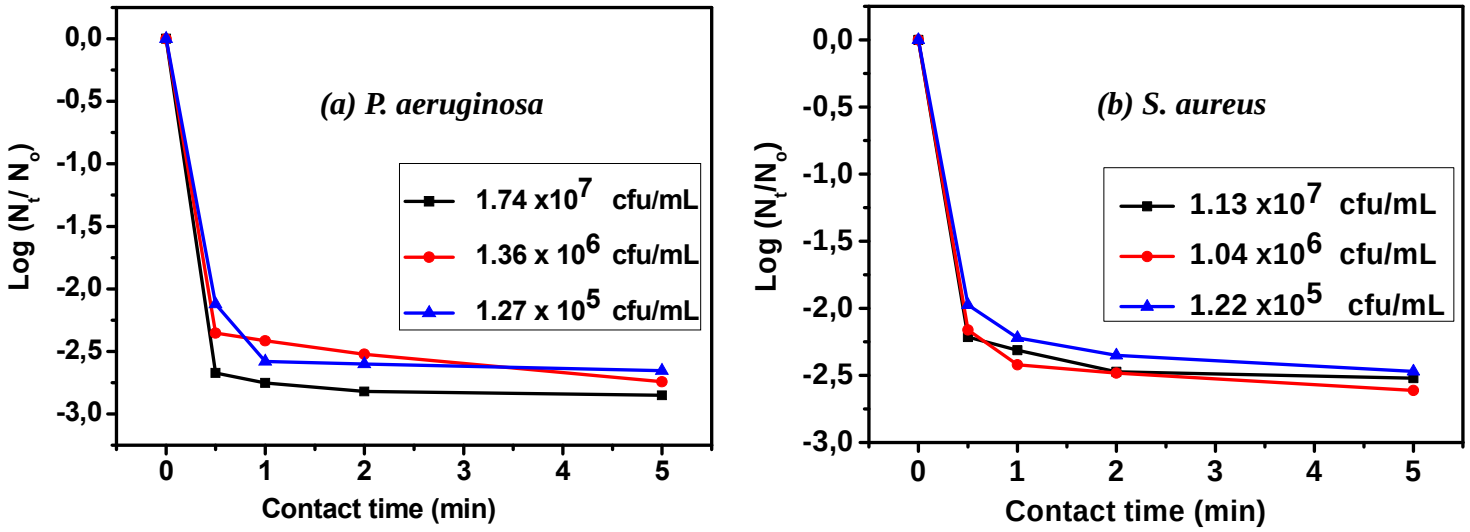

Figure 4.4 Kinetics of chlorine dioxide inactivation of bacteria at varying initial densities. (a) *P. aeruginosa* exposed to 1.0 mg/L and (b) *S. aureus* exposed to 2.5 mg/L ClO_2

4.3.5 Permeability of the outer cell membrane.

The potential of chlorine dioxide to compromise the integrity of the outer cell membrane and its contribution to the bactericidal mechanism was assessed by the NPN uptake assay. The outer cell membrane is a distinguishing feature of Gram-negative bacteria[59] and hence the NPN assay was conducted only with *P. aeruginosa*. In Table 4. 2, the effect of chlorine dioxide on the NPN uptake assay by *P. aeruginosa* cells is illustrated. Exposing the cells to chlorine dioxide

resulted in a substantial increase in the uptake of the chromogenic probe NPN in a concentration-dependent manner. For instance, the treatment of cells with 1.0 mg/L, 2.5 mg/L and 5.0 mg/L concentrations of chlorine dioxide yielded mean uptakes of approximately 17%, 47%, and 83% respectively of the uptakes induced by 1.0 M EDTA which is a well-known membrane permeabilizer.

Table 4. 2. NPN uptake by *P. aeruginosa* cells after treatment with chlorine dioxide

Sample	NPN	Maximum fluorescence (Mean ± SD)	% NPN Uptake
HEPES Buffer (5 mM, pH 7.4)	**-**	**78 ± 2.9**	
Cell suspension only	**+**	**204 ± 17.6**	
Cell suspension+ EDTA (1.0 mM)	**+**	**613 ± 13.4**	**100**
Cell suspension + ClO_2 (1.0 mg/L)	**-**	**227 ± 8.7**	
Cell suspension + ClO_2 (1.0 mg/L)	**+**	**275 ± 9.2**	**17.4**
Cell suspension + ClO_2 (2.5 mg/L)	**-**	**237 ± 5.9**	
Cell suspension + ClO_2 (2.5 mg/L)	**+**	**397 ± 13.1**	**47.19**
Cell suspension + ClO_2 (5.0 mg/L)	**-**	**283 ± 15.2**	
Cell suspension + ClO_2 (5.0 mg/L)	**+**	**543 ± 21.1**	**82.9**

The outer leaflet of the outer membrane of *P. aeruginosa* is principally made of lipopolysaccharides and functions as an effective permeability barrier to external hydrophobic or lipophilic molecules including NPN[60]. However, when the integrity of the outer membrane is altered by external agents such as membrane-permeabilizing compounds, the NPN molecules permeate into the phospholipid environment of the inner leaflet of the outer membrane to produce an enhanced fluorescence intensity whose magnitude is dependent on the quantity of molecules of the probe entering the cells.

On the contrary, NPN molecules in the external aqueous environment of cells tend to fluoresce weakly. The present results in Table 4. 2, indicate that the exposure of cells to chlorine dioxide disrupted the intact external lipopolysaccharide membranes and enabled the entry of the chromogenic NPN molecules[35]. This resulted in the enhanced fluorescence intensity expressed as uptake. Increasing the concentration of the chlorine dioxide also led to a higher NPN uptake which might indicate an increase in the number of bacterial cells whose outer membranes were altered. An alteration of the membrane might thus correspond with an increase in cell inactivation or death.

4.3.6 The release of DNA, s and RNA, s from cells.

The bacterial cytoplasmic membrane is a site for vital cellular activities such as respiration, synthesis of lipids and cell wall constituents, maintenance of the homeostatic conditions as well as the control of several metabolic processes of the cell[59]. It is principally made of a semi-permeable phospholipid bilayer with embedded proteins and regulates the transfer of solutes and metabolites in and out of the cell. Compromised cytoplasmic membrane results in the discharge of critical intracellular cell components such as nucleic acids (DNA and RNA) which absorb UV- light strongly at 260 nm into the surrounding matrix[61].

In Figure 4.4, the plots indicate the release of nuclear materials following the exposure of cell suspensions to varied concentrations of chlorine dioxide. The absorbance curves illustrate the ratio of chlorine dioxide treated cells to the untreated cells used as a negative control and are generally characterised by a sharp increase within the initial 10 min of measurement and essentially becoming steady afterward. At the 5.0 mg/L dose, nearly 1.8 and 1.6-fold increases in the 260 nm absorbing materials were observed for *P. aeruginosa* and *S. aureus* respectively

at 40 min of contact. The quantity of the intracellular nuclear materials released into the extracellular matrix was also observed to increase with the initial concentration of chlorine dioxide and this trend was evident in both organisms. A strong correlation (supporting information S4.1) between the maximum level of inactivation obtained for a given concentration and the maximum 260 nm absorbing materials released as determined by the absorbance measurements was also observed.

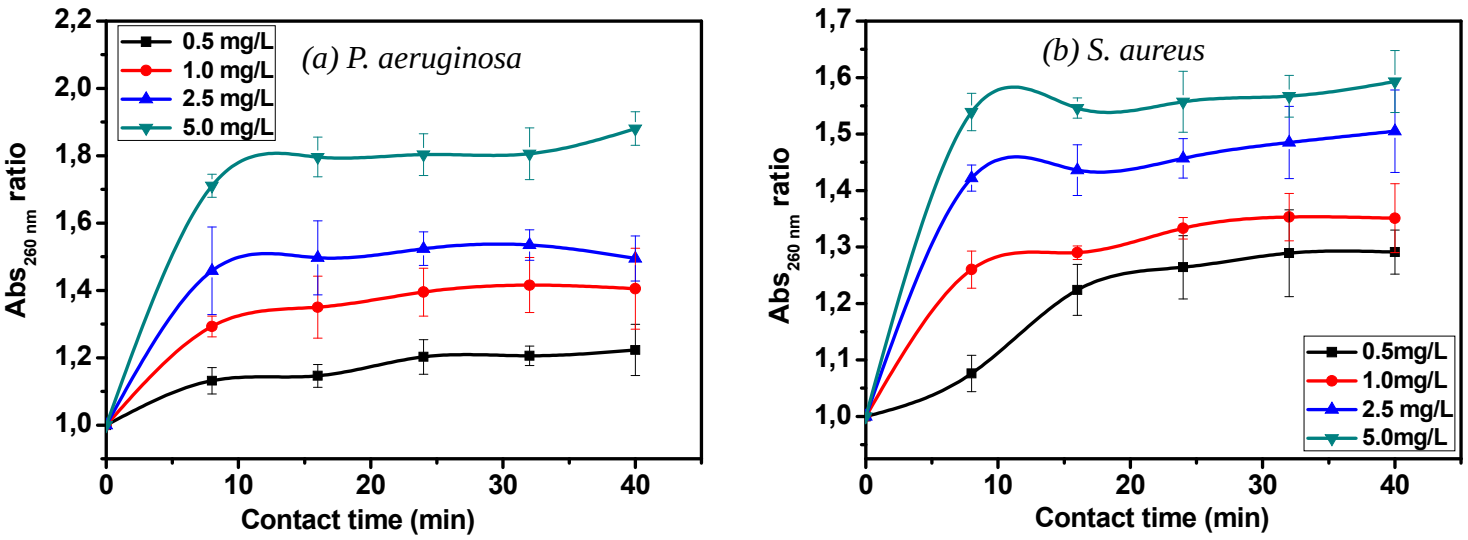

Figure 4.4 Effect of ClO_2 treatment on the release of 260 nm absorbing materials from (a) *P. aeruginosa* and (b) *S. aureus*. Absorbance data expressed as a ratio of the ClO_2 treated cells relative to the untreated cells.

Chlorine dioxide is an oxidant and inactivates microbial cells by oxidation. Its reactions proceed by the abstraction of a single electron from the reactive organic substrates of the bacterial cells. Possible sites for initiating the oxidation reactions depend on the selective reactivity of chlorine dioxide towards organic functional groups or compounds. For instance, chlorine dioxide reacts slowly or is virtually unreactive with primary amines and unsaturated fatty acids[62]. However, the reactions with amino acids such as cysteine, tyrosine, and tryptophan have been found to be rapid[47] and are therefore considered as favourable reactive

sites for initiating reactions with the cytoplasmic membrane. Chlorine dioxide has also been demonstrated to undergo extremely fast oxidation reactions with nicotinamide adenine dinucleotide (NADH) which is a key coenzyme in many biological redox reactions as well as the synthesis of adenosine triphosphate (ATP)[63].

Linking these results, suggest that after permeating through the outer membrane of a bacterial cell, chlorine dioxide further targets the cytoplasmic membrane and oxidises by redox reactions with more reactive reducing species in the membrane. It is most unlikely that reactions with a specific target group of compounds or components of the cell might be responsible for the cell death. Instead, the reactions with multiple targets within the cell might eventually accumulate to impair on vital metabolic processes and resulting in loss of activity or death.

4.3.7 Changes in cell morphology

The effect of chlorine dioxide on the morphology of bacterial cells was assessed by TEM. Representative electron micrographs showing the morphologies of *S. aureus* and *P. aeruginosa* cells before and after treatment are displayed in Figure 4. 5. Typical cocci -shaped cells with well-defined intact inner and outer membranes were observed from the images of *S. aureus* before and after treatment. Visible morphological damages to the cell structure by chlorine dioxide were not prominent. However, some apparent increase in the roughness and small indentations on the outer cell surfaces (as indicated by arrows) were seen for the treated *P. aeruginosa* cells.

Further analysis of the images also showed a significant increase ($p < 0.05$) in the thickness of the cell wall of *P. aeruginosa* after the treatment with chlorine dioxide (mean thickness before treatment was determined as 33.85 ± 5.30 nm and after treatment was 55.43 ± 6.58 nm). On the other hand, the thick peptidoglycan cell walls of Gram-positive *S. aureus* might have enhanced the tolerance of the cell wall to visible damages. That notwithstanding it is evident in these observations that chlorine dioxide does not oxidise or inactivate bacteria cells by inflicting gross morphological damages. It is even more interesting to reconcile how it induces an increase in the permeability of both the outer cell and the cytoplasmic membranes without necessarily causing cell lysis. Chlorine dioxide is a highly selective monomeric free radical[58] that has the potential of diffusing through the membranes and reacting with the more reactive moieties of the cell constituents such as glutathione, tryptophan, and cysteine. The efflux of the vital cellular components may significantly contribute to the overall cell death.

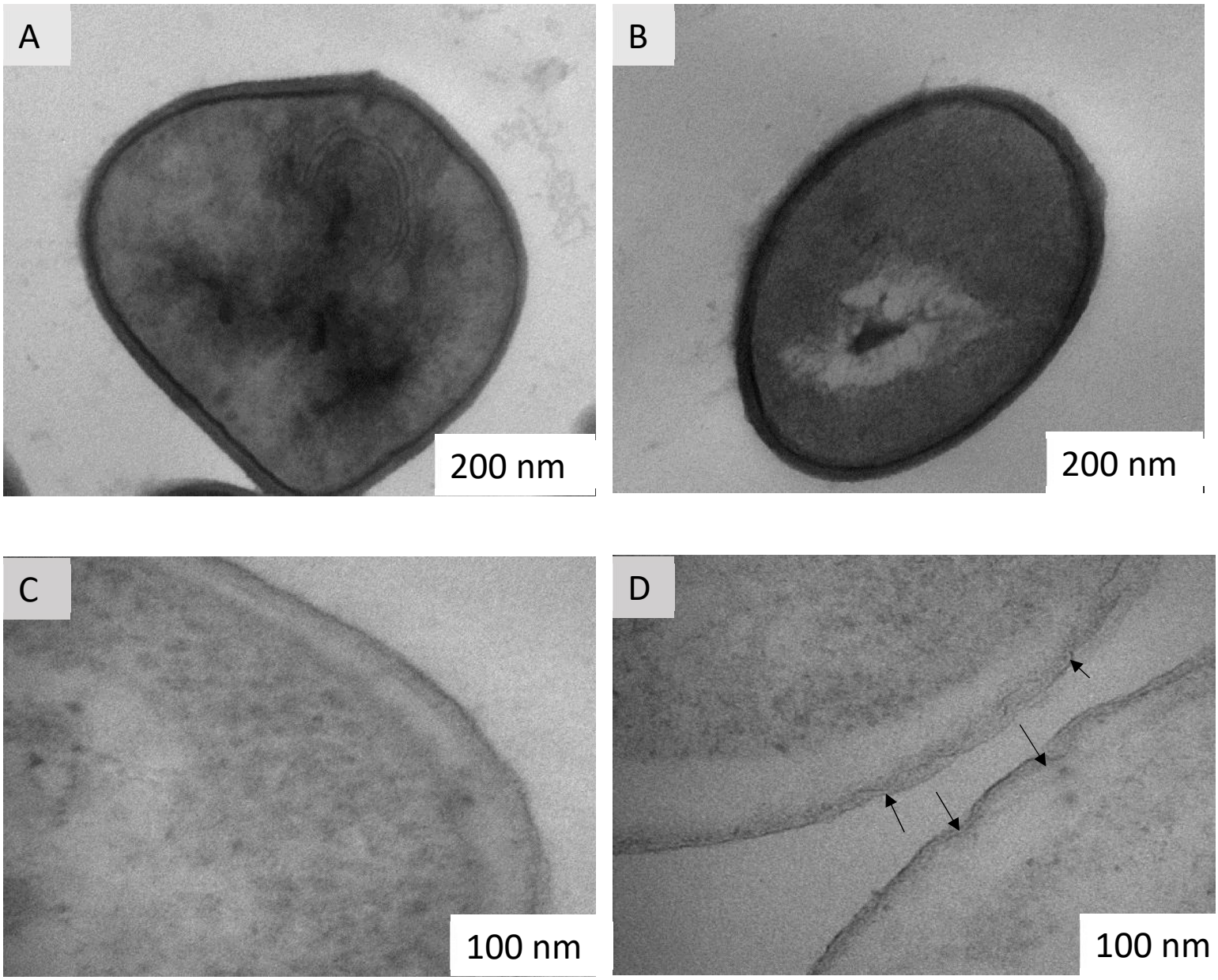

Figure 4. 5. TEM micrographs of bacteria cells before and after treatment with 4.0 mg/L ClO_2, (22 ± 2 ºC, pH 8.0). (A) *S. aureus* before treatment (B) *S. aureus* after treatment (C) *P. aeruginosa* before treatment (D) *P. aeruginosa* after treatment

4.4 Conclusion

In this study, the kinetics of chlorine dioxide as a disinfectant for the control of *P. aeruginosa and S. aureus* were monitored. The results have shown that chlorine dioxide at 5.0 mg/L is suitable for eliminating potential threats of *P. aeruginosa* and *S. aureus* in water. However, at the maximum doses (1.5 mg/L) recommended for drinking water, a substantial number of bacteria population in a highly contaminated water could still survive the inactivation of chlorine dioxide. Chlorine dioxide is more efficient as a disinfectant at higher pH most especially in the alkaline conditions, but its rate of disinfection is less impacted by the initial density of bacteria. Chlorine dioxide does not kill bacteria cells by lysing but rather diffuses through the outer and cytoplasmic membranes, increases their permeability and cause the release of vital cell components and essentially leading to cell death.

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Supporting Information

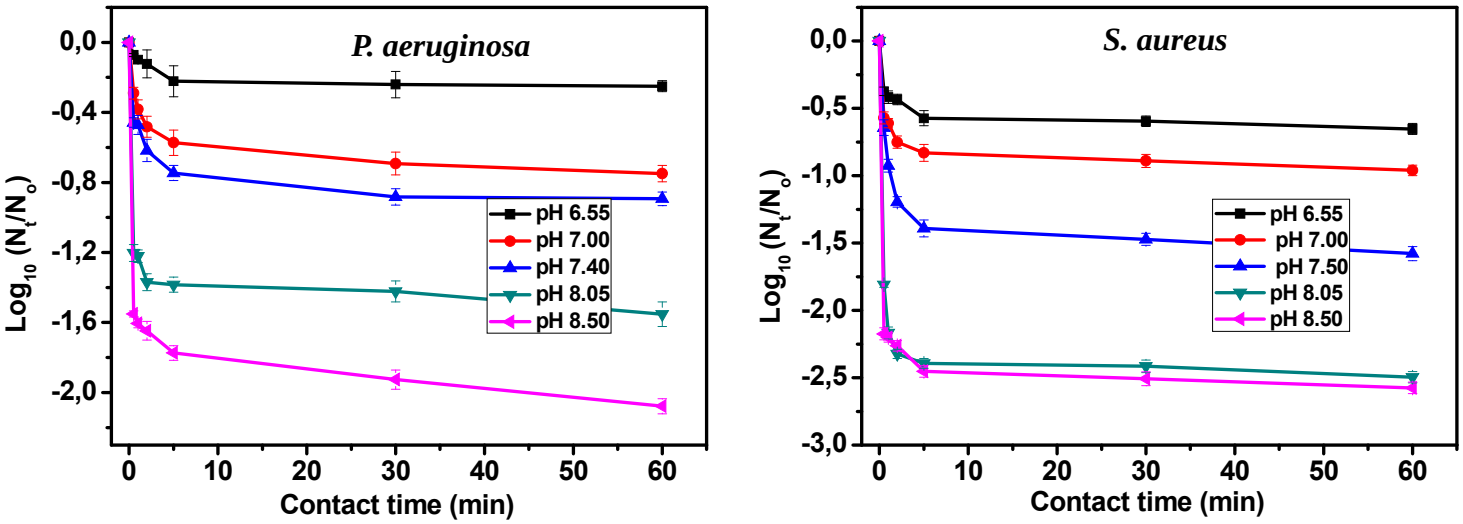

Figure S4.1. Inactivation kinetics of *P. aeruginosa* and *S. aureus* exposed to 2.0 mg/L chlorine dioxide at varied water pH conditions

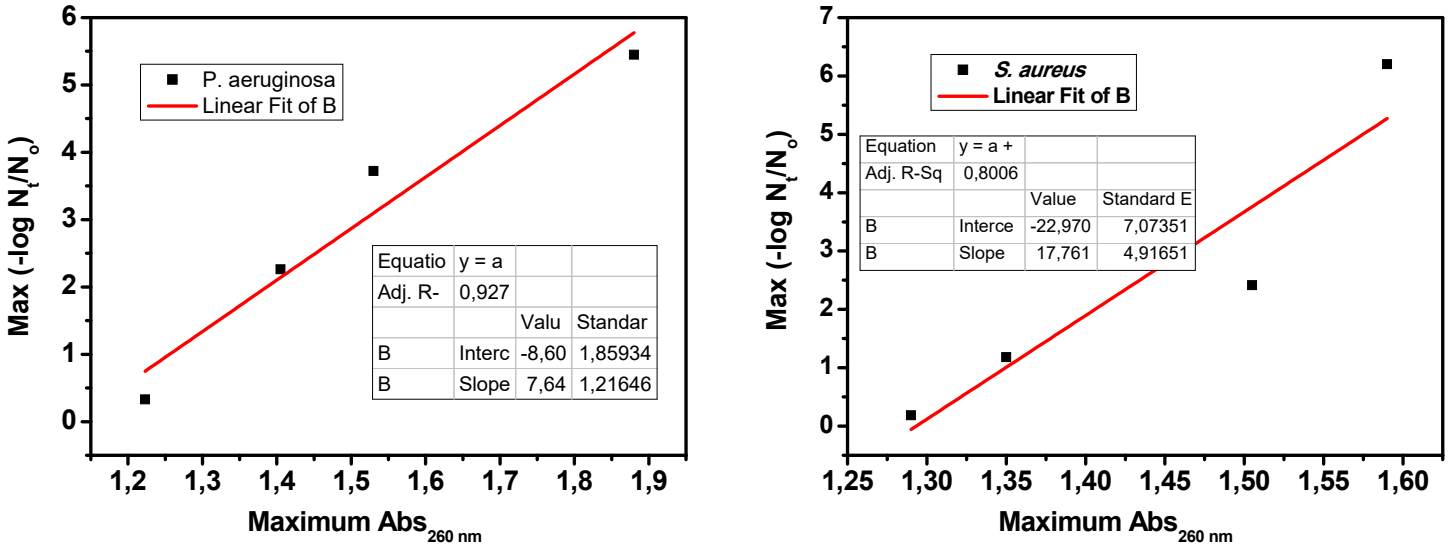

Figure S4.2. A plot showing the correlation between the maximum level of inactivation and the maximum absorbance of 260 nm absorbing materials of *P. aeruginosa* and *S. aureus* exposed to 5.0 mg/L chlorine dioxide

CHAPTER 5

Profiling the Susceptibility of Autochthonous Wastewater Bacterial Community to Chlorine dioxide disinfection by Polymerase Chain Reaction-based Denaturing Gradient Gel Electrophoresis

Abstract

Bacterial populations in environmental water systems are diverse and exhibit different responses to water disinfectants. In this study, the susceptibilities of autochthonous bacterial community from a typical municipal wastewater towards varying concentrations of chlorine dioxide (0.5 – 5.0 mg/L) was demonstrated by 16S rRNA gene directed PCR-DGGE as a culture-independent technique in comparison to classical heterotrophic plate count culture-based methods. Bacteria exposed to chlorine dioxide were pre-treated with propidium monoazide (PMA) to isolate the DNA of viable cells from membrane-compromised cells. Significant differences in bacterial susceptibilities were observed between the two techniques. A 3.0 mg/L chlorine dioxide dose, sufficiently eliminated the heterotrophic bacteria population to achieve an approximate 4.0 ± 1 log reduction in just 30 s from the culture-based protocol. On the contrary, the PCR-DGGE profile rather showed that 1.0 mg/L was adequate to inactivate three predominant species identified as *Arcobacter suis F41*, *Pseudomonas sp strain QBA5* and *Pseudomonas sp B-AS-44*, whereas a significant population of other species such as *Pseudomonas sp CCI2E* were observed to presumably remain viable to 5.0 mg/L chlorine dioxide. It is envisaged that the results of this study will broadly influence the monitoring strategies used for assessing the dose-response effect of a disinfection regime and prospecting for potential organisms that might be resistant towards water disinfectants.

Keywords: PCR-DGGE; Propidium monoazide; chlorine dioxide; Wastewater; Disinfection.

5.1 Introduction

The treatment and reuse of wastewater or greywater are currently at the forefront of the scarce water resource management strategies globally. Reclaimed water when effectively treated could be utilised for several purposes including agricultural irrigation, recreational and environmental supply, recharge of surface and ground waters, domestic toilet flushing and various industrial applications[1]. In some instances, it could be treated to drinking water standards and supplied into taps. The prevention of infectious diseases by pathogenic agents in reclaimed water is one of the key issues considered when evaluating the quality and efficiency of the treatment process. In most cases, it is achieved during the disinfection stage which is usually the final step in the treatment process prior to being discharged into the distribution system or receptor water bodies.

Conventionally, the reliability of a disinfection performance at a treatment plant is validated on indicator organisms, which usually include total coliforms, faecal coliforms, and coliphage[2-4]. Such monitoring processes commonly utilise culture-dependent techniques including the heterotrophic plate count (HPC) to assess microbial viability and to determine the effectiveness of the disinfection activity. Culture-based methods are relatively simpler, cost-effective in terms of equipment and other logistics, do not usually require high technical expertise and more suitable for routine monitoring.

However, it is estimated that more than 99% of the bacterial diversity in oligotrophic habitats like disinfected wastewater may be non-cultivable or present in viable but non-culturable (VBNC) states[5-7]. It follows therefore that considerable details about most of the diverse bacteria community and their susceptibility to disinfectants in the water system may not be known, thus underestimating the number of pathogenic microbes that remain viable after disinfection[8]. Another limitation is the inability of microbial indicators to adequately predict the presence of some pathogens. For instance, coliform bacteria are relatively more susceptible to chemical disinfection and hence may not adequately reflect the occurrence of pathogens in disinfected reclaimed water containing protozoan parasites such as Cryptosporidium and enteric viruses[9].

There has been a growing number of research findings in recent times on wastewater facilitated development and transfer of clinically relevant antibiotic-resistant bacteria (ARB) and antibiotic resistant genes (ARG)[10-13]. These have partly been attributed to inadequate disinfection emanating from the exposure to subinhibitory concentrations of various disinfection technologies including chlorination and UV irradiation leading to the triggering of biochemical stress responses[14-15]. Due to the complexity of ARB and ARG as emerging contaminants in wastewater and their associated challenges, the application of adequate dosages of a disinfectant and the validation of its adequacy are critical for preventive and corrective actions. An integrated approach to monitoring the efficiency of a disinfection regime and prospecting for potential disinfectant resistant strains of bacteria at a treatment plant might essentially require some culture-independent techniques to occasionally profile the changes in the community structure and monitor the disinfection efficiency.

Culture-independent techniques such as 16S rRNA gene directed polymerase chain reaction (PCR) based denaturing gradient gel electrophoresis (DGGE) presents an invaluable tool to study the dynamics of an unknown diverse bacteria populations and their responses to a disinfectant. DGGE is a molecular fingerprinting technique that separates double-stranded DNA (dsDNA) fragments from PCR products of similar length but of different base pair sequences[16]. The separation is based on the distinct mobilities of partially melted DNA in a polyacrylamide gel along with a linear gradient of DNA denaturants (usually urea and formamide). Most previous applications of the DGGE technique in water treatment processes focussed mainly on monitoring the long-term changes in bacterial community structures of either an environmental water body[17] or in a drinking water distribution systems[5, 18]. Moreover, the limited reports in the literature regarding the use of culture-independent methods to assess inactivation efficiencies of water disinfectants were mainly directed towards specific target organisms[8, 19]. However, in a complex system such as a municipal wastewater, diverse bacteria species with different susceptibilities or responses towards a disinfectant may exist.

Chlorine dioxide (ClO_2) is one of the chemical alternatives to conventional chlorination for the disinfection of wastewater effluents[20]. It has a unique advantage of not forming organic disinfection products such as trihalomethanes or haloacetic acids, most especially when used as a pre-oxidant during water treatment. It also oxidises microelements such as iron (Fe^{2+}) and

manganese (Mn^{2+}) into insoluble forms (Fe^{3+} and Mn^{4+}) that may easily be filtered off prior to discharge[21]. Chlorine dioxide is also known to possess broad-spectrum biocidal activity against a range of microbial organisms including bacteria, viruses, and protozoa[22].

In this study, we explored the PCR based DGGE as a culture-independent technique to investigate the susceptibilities of autochthonous bacterial community in an urban wastewater to varying concentrations of ClO_2 and compared with the heterotrophic plate count method.

5.2 Materials and Methods

5.2.1 Preparation of chlorine dioxide

Chlorine dioxide solutions were prepared as described previously [4] by oxidising approximately 25% (w/v) solution of sodium chlorite ($NaClO_2$) in a gas generating bottle with a dilute solution of sulphuric acid (H_2SO_4, 2M). The generated gas was harvested through a stream of compressed air into a connecting chlorine scrubber system, which contained a saturated solution of sodium chlorite (10% w/v) to scrub contaminants such as chlorine gas. Chlorine dioxide gas was collected in a connecting bottle of demand free deionized water and the concentrations of the prepared stock solutions were analysed by the Iodometric method whilst the residual concentrations were determined by the N, N-diethyl-p-phenylenediamine (DPD) method[4].

5.2.2 Water sampling and determination of physicochemical parameters

Samples of untreated influent wastewater were collected from the eThekwini Wastewater Treatment Plant (EWWTP) in Durban, South Africa according to common sampling protocols [4], kept on ice and immediately transported to the laboratory for further analysis. For any experimental event, 5.0 L composite samples were prepared by mixing the contents of the sampling bottles to obtain a uniform homogeneous matrix. Samples were filtered with Whatman No 1 filter papers (pore size 11µm) to remove particulate matter and subsequently determining the pH (Beckman pH meter, CA, USA), total dissolved solids (TDS) and Electrical conductivity (EC) using the CD401 probe fitted onto HQ40d multimeter (HACH, Co, USA). The COD and total suspended solids (TSS) were determined according to Standard Methods for the examination of water and wastewater[4].

5.2.3 Disinfection of water samples with chlorine dioxide

Approximately 500 mL portions of the filtered water samples were exposed to different concentrations (0.5 to 5.0 mg/L) of chlorine dioxide at 22 ± 2 °C in 1.0 L batch reactors under constant agitation on a magnetic stirrer. These concentrations were considered in accordance with typical doses employed for water disinfection[23-24]. The inactivation kinetics of the autochthonous bacteria was monitored by withdrawing 5.0 mL of the samples from the reactor at predetermined time points into tubes containing 2 mL of 10 mM sodium thiosulphate in each case to quench the residual chlorine dioxide. Total heterotrophic cultivable bacteria populations were subsequently enumerated in triplicates on tryptone soy agar (TSA) by the spread plate count technique (upon appropriate dilution) following incubation at 37 °C for 18 - 24 h. Processing of each sampled water was conducted within 24 h after collection. Control samples were identically treated except that no chlorine dioxide was added.

5.2.4 Propidium monoazide treatment and metagenomic DNA extraction

To isolate the DNA of viable cells from the dead cells after chlorine dioxide treatment, a protocol involving the application of propidium monoazide (PMA) [Phenanthridiun, 3-amino-8-azido-5-[3-(diethylmethylammonio) propyl]-6-phenyl dichloride] as described previously [25-26] but with some modifications was employed. Chlorine dioxide disinfected wastewater samples corresponding to each applied concentration in the reactor were quenched with 10.0 mL of 10 mM sodium thiosulphate after incubating for 1 h. Samples were centrifuged in 250 mL centrifuge bottles at 10000 *x g* and the pellets washed and concentrated to a total of 1.0 mL in microcentrifuge tubes with autoclaved deionized water. Desired quantities of PMA (Biotium Inc, Hayward, CA, USA) sufficient to give an approximate final concentration of 50 µmol/L from a prepared stock of 2.0 mmol/L were added to each of the aliquots in the tubes, mixed thoroughly and incubated in the dark for 20 min. Subsequently, samples were placed horizontally on ice and exposed to light from a halogen lamp (26W, OSRAM DULUX, China) from an approximate distance of 30 cm for 10 min, vortexed thoroughly and the contents transferred directly into the 5.0 mL bead beating tubes provided in the PowerWater DNA Isolation kit (MO BIO Laboratories, USA). The total metagenomic DNA was extracted according to the manufacturer's instructions and stored at -20 ºC for further molecular analysis.

Purified DNA extracts were assessed by agarose gel electrophoresis [27] whilst the quantity and quality was verified with a NanoDrop 2000C Spectrophotometer (Thermo Scientific, USA)

5.2.5 Amplification of 16S rRNA gene by PCR using DGGE primers

A polymerase chain reaction (PCR) was performed to amplify a 586 bp universal 16S rRNA gene fragments using the universal forward primer 341F (5′CCT ACG GGA GGC AGC AG 3′) and the reverse primer 907R (5′ CCG TCA ATT CMT TTG AGT TT 3′). A GC – clamp (CGC CCG CCG CGC CCC GCG CCC GTC CCC GCC CCC GCCC) which has a high melting domain was attached to the 5′ end to prevent a complete denaturation of DNA fragments and hence enabling the detection of the corresponding PCR product during the DGGE [28]. The PCR mixture (50 µL) composed of 25 µL of 2 x Phusion flashhigh-fidelity PCR master mix (Thermo Scientific), 0.4 µM of each primer and about 10 – 30 ng µL of genomic DNA. The reaction was conducted in an automated thermal cycler (T100™ Bio-Rad, USA) under a touchdown PCR program as follows: initial denaturation at 94 °C for 5 min, followed by 20 cycles of 94 °C for 1 min, 65 °C for primer annealing for 1 min and 72 °C for 1 min. The annealing temperature was decreased by 0.5 °C per cycle until a touchdown at 55 °C. This was followed by another 15 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 3 min and a final extension at 72 °C for 7 min before holding at 4 °C [16]. Negative control reactions in the absence of the DNA template were run simultaneously.

5.2.6 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was carried out on a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, USA) following the manufacturer's instructions. In this protocol, approximately 1000 ng of PCR amplicons were loaded onto a vertical polyacrylamide gel (6% w/v) in a 1 x TAE buffer using a denaturing gradient ranging from 40 to 60% (100% denaturant solution contained 7 M urea and 40% deionized formamide)[5]. Electrophoresis conditions were set at 60 V to run for 16 h, following which the gel was stained with ethidium bromide for 30 min and de-stained in 1 x TAE buffer for 15 min. The gel was subsequently visualized and imaged under UV light with a Gel Doc system (Syngene, UK).

Bright representative bands were excised into sterile 1.5 mL Eppendorf tubes to elute the DNA in an elution buffer after being kept overnight at 4 ºC. Essentially, the eluted DNA samples were re-amplified with the primers previously described but without the G-C clamp under identical PCR conditions. The amplicons were sequenced (Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa) and the sequences edited with Chromas (Technelysium Pty Ltd, Brisbane, Australia) and then compared against the NCBI non-redundant database using the basic local alignment search tool (BLAST) to reveal their identity.

5.2.7 Data Analysis

The data from the inactivation of the heterotrophic bacteria count were described by the C_{avg} Hom model (CaH) previously used for describing disinfection kinetics data often characterised by a tailing off behaviour [29-30].

$$\log (N_t / N_0) = -kC^n_{avg} T^m \quad (1)$$

$$C_{avg} = \sqrt{C_0 . C_f} \quad (2)$$

where C_0 and C_f are the initial and final disinfectant concentrations (mg/L) respectively, (N_t/N_0) is the survival ratio of number of organisms surviving at time, t (N_t) and at time, t = 0, N_0 (cfu/mL)), *k* is inactivation rate constant of the target organism, T is the contact time required to achieve a given level of inactivation, *n* is an empirical factor called the *coefficient of dilution,* whilst *m* is an empirical constant.

5.3 Results and Discussion

5.3.1 Inactivation kinetics of wastewater total heterotrophic bacteria

The present study aimed at determining the susceptibilities of wastewater bacteria community to different concentrations of chlorine dioxide applied as a disinfectant by using both culture-dependent and independent techniques such as PCR-DGGE. Figure 5.1 is an illustration of the inactivation kinetics data of chlorine dioxide-initiated disinfection of typical municipal wastewater samples at different doses. A summary of the measured physicochemical parameters of the wastewater samples is also shown in Table 5.1. Total heterotrophic bacteria densities ranged from 10^4 to 10^5 cfu/mL. The application of 0.5 mg/L of chlorine dioxide

resulted in ~ 0.3 log reduction of the bacterial population within 1 hour of contact time whilst approximately 2.0 log reduction was achieved after treating the water samples to 1.0 mg/L. Meanwhile, an increase of the chlorine dioxide concentration to 3.0 mg/L was sufficient to eradicate almost completely all the heterotrophic bacteria to below detection limits to yield more than 4 log inactivation of the wastewater autochthonous bacteria. This observation was consistent in almost all samplings and independent experimental events conducted and agreed well with the results of other similar studies [31-33].

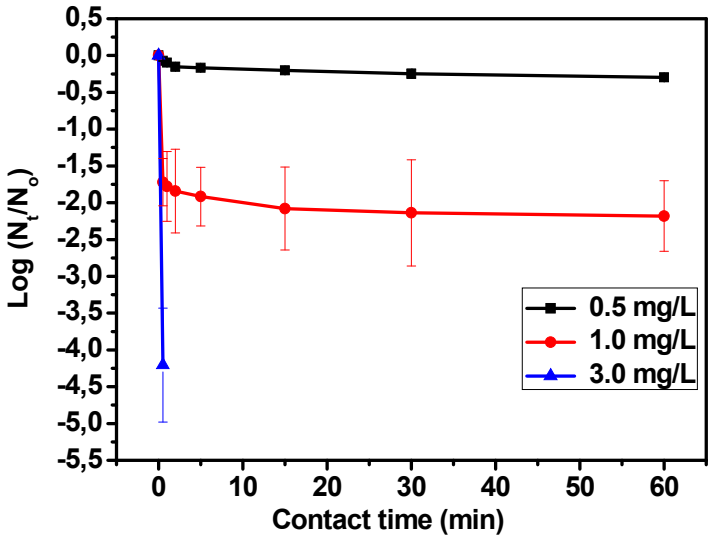

Figure 5.1 The inactivation kinetics of heterotrophic bacteria from wastewater samples by chlorine dioxide applied at different concentrations.

The findings demonstrate the exceptional efficiency of chlorine dioxide as a disinfectant of choice for the treatment of wastewater. This characteristic can presumably be attributed to the fundamental selective reactivity of chlorine dioxide towards organic compounds in a typical water application [23, 34]. Generally, when compared to chlorine, it is less reactive towards compounds containing olefinic double bonds, primary and secondary amines, aromatic hydrocarbons, aldehydes and ketones, carbohydrates [35-36] as well as humic and fulvic acids [37]. These results in a lesser disinfectant demand for the applied dose and eventually making it more available for microbial inactivation.

Table 5.1. Selected Physicochemical parameters determined from the influent wastewater samples of the eThekwini Waste Water Treatment plant, Durban

pH		7.35 ± 0.4
EC	µS/cm	1128 ± 31
Turbidity	NTU	32.8 ± 4.1
COD	mg/L	112 ± 8.7
TSS	mg/L	67.3 ± 5.4

Values are averages of triplicate measurements ± standard dev (SD)

5.3.2 PCR-DGGE profile of bacterial community in wastewater exposed to chlorine dioxide

The amplified DGGE-PCR products of the extracted metagenomic DNA from the bacterial community in the sampled municipal wastewater after chlorine dioxide and PMA treatments are depicted in an agarose gel in Figure 5.2.

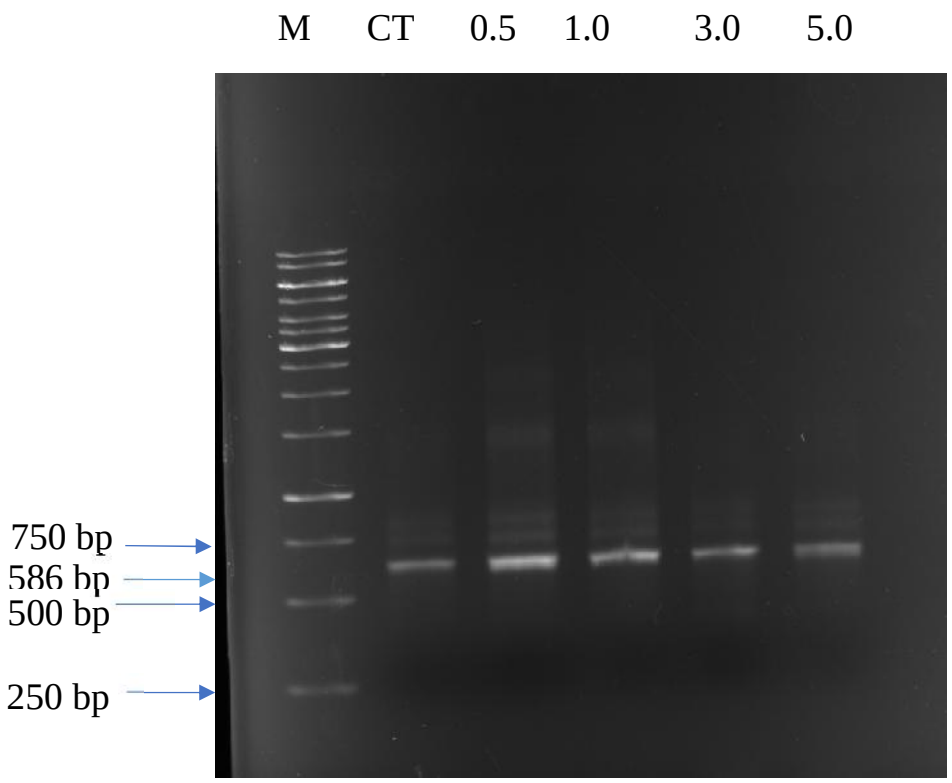

Figure 5.2 Representative agarose gel showing the PCR amplicons of the extracted bacterial DNA from a wastewater sample using 341F-GC and 907R primers. M contains a 1 kb marker (Thermo scientific), CT represents the control sample (without ClO_2 treatment) and the rest depict the concentrations of chlorine dioxide (mg/L) applied to disinfect the water samples

Figure 5.3 shows the DGGE profile of the bacterial community in the wastewater sample exposed to varying concentrations of chlorine dioxide. Each band on the profile is assumed to represent a different operational taxonomic unit (OTU) corresponding to a single species. Bands appearing to be common to each of the lanes also indicate the presence of common species in the analysed samples. Moreover, under the same experimental conditions, the relative intensities of the bands are also assumed to correspond to the relative abundance of the diverse species present.

Prior to the DNA extraction, cells were exposed to PMA to selectively isolate the DNA of the dead cells from the viable cells for the essential downstream analysis [38]. PMA is a high-affinity photo reactive DNA binding dye which is impermeable to intact cells but readily permeates through compromised cell membranes to intercalate with the inner or naked DNA found in the debris of lysed cells. This reaction forms an irreversibly modified DNA complex that inhibits

the subsequent PCR amplification of the DNA templates of dead cells[26]. It implies, therefore, that; the visible bands on the gel could reliably indicate the bacterial cells that survived the doses of chlorine dioxide.

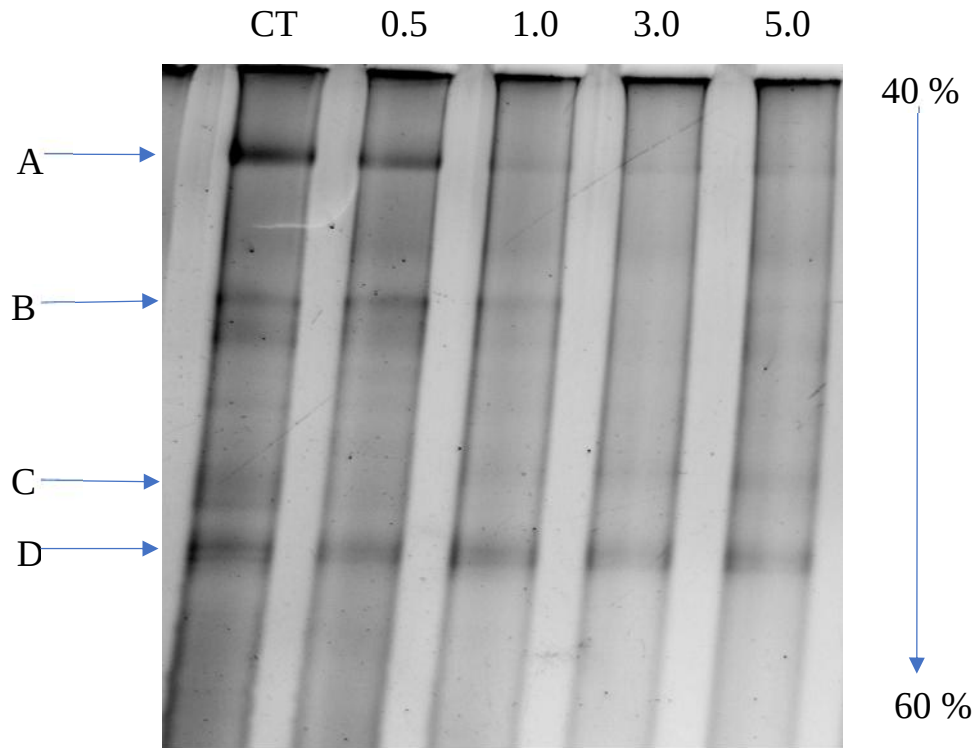

Figure 5.3 A DGGE profile of the bacterial community in the sampled wastewater exposed to varying concentrations of chlorine dioxide. The lane labeled CT represents the control (without ClO_2 treatment), whilst the other lane labels depict the concentrations of chlorine dioxide (mg/L) applied to disinfect the water. The A-D labeling shows the dominant OTUs excised for sequencing

The lane labeled CT represents the original strength of the bacteria population in the wastewater samples prior to the treatment with ClO_2. Meanwhile, in Figure 5.3. the bright representative bands of each class, which were excised and sequenced are labeled A-D. The OTU labeled as A, appeared more intense in the control sample CT, and at 0.5 mg/L concentration. This intensity was observed to decline from 1.0 mg/L through to 5.0 mg/L,

indicating a substantial reduction in the population of the viable bacteria species to below detectable levels. Similar observations were also made for B, whilst the band class ascribed as C was only visible in the CT sample. These observations suggest that a chlorine dioxide concentration of 1.0 mg/L was sufficient to inactivate the bacteria species in the wastewater samples represented by bands A, B, and C on the gel.

However, quite interestingly, relatively brighter or more intense bands were observed for the OTU labeled D in the CT and all the other samples subjected to the varying chlorine dioxide concentrations. These bands represent dominant species of bacteria in the composite wastewater whose population could not be substantially degraded to below detectable limits in the gel even at 5.0 mg/L. It thus indicates that such species are relatively less susceptible to chlorine dioxide and their abundance in the composite water samples essentially correspond to the intensity of the bands in the gels.

It is also worth noting that the inactivation data from the culture-based heterotrophic plate count technique (Figure 5.1) showed that at 3.0 mg/L, all the cultivable bacteria on the tryptone soy agar (TSA) plates were effectively inactivated within the initial 30 s. This shows that the dominant species found in the OTU labeled D could not have been present as detectable colonies on the TSA plates at 3.0 mg/L or higher concentrations thereof, but they could, however, be detected on the DGGE fingerprint profile. Such species could be viable but non-culturable (VBNC) or non-viable but still possess intact membranes [18]. Recently, it has been reported that the exposure to low concentrations of oxidants such as chlorine, monochloramine, and ozone-induced VBNC states to *E. coli*, *Salmonella*, and *Legionella* spp and enhanced their reactivation and regrowth, or the persistence and resistance towards antibiotics [1, 39-40].

A key assumption underlying the PMA treatment stage in this analysis is that cells with compromised or injured cell membranes are assumed to have lost their viability [41]. However, in relying absolutely on the membrane integrity for viability and consequent efficiency of a disinfection system as in this study, knowledge about the bactericidal mechanism of the disinfectant becomes necessary. In a recent work conducted by our research group [42], chlorine dioxide was found to principally inactivate *E. coli* by disrupting the integrity of the outer cell

and the cytoplasmic membranes to release intracellular components without necessarily lysing the cells. On the contrary, the primary bacterial inactivation mechanism of UV- light involves the damage of the DNA without necessarily compromising the integrity of the outer cell membrane [18, 43]. In such a case, membrane integrity becomes a poor indicator of cell viability.

5.3.3 Identities of dominant species from the DGGE profile

The identified dominant species of bacteria based on the BLAST comparison of sequences against the NCBI Genbank database is summarised in Table 5.2. The OTU labeled A was found to possess 99% similarity to the 16S rRNA sequence of *Arcobacter suis* F41. The *Arcobacter* species have been found to be highly abundant in sewage, raw (untreated) environmental waters as well as secondary effluents where they are estimated to constitute approximately 5 – 11 % of the bacteria population in such habitats [44-45]. Even though no known resistance of the *Arcobacter sp.* to common chemical-based water disinfection technologies such as chlorination, chlorine dioxide, monochloramine or ozone have been reported in the literature, some strains of clinical relevance such as *A. butzleria* have been identified to be resistant to ampicillin and cefotaxime [46].

On the other hand, the other OTUs, B, C, and D were predominantly similar in identity to different strains of *Pseudomonas* species with marked susceptibility differences towards chlorine dioxide. For instance, whilst 1.0 mg/L chlorine dioxide was sufficient to completely reduce B (*Pseudomonas sp* strain QBA5) and C (*Pseudomonas sp* B-AS-44), a concentration of 5.0 mg/L was not enough to effectively remove D (*Pseudomonas sp* CC12E). However, among the non-fermenting Gram-negative bacilli, *P. aeruginosa* is the most prevalent species of clinical significance and thrives in diverse environments including soil, water, and surfaces of medical equipment [47].

The concerns of interest in this circumstance lie with the potential of the presumed chlorine dioxide resistant gene elements embedded in strains such as *Pseudomonas sp* CC12E to be horizontally transferred across into virulent strains of *P. aeruginosa* in wastewater systems [48] and potentially to other bacteria. The consequent health implications associated with their infections most especially among immunocompromised patients could be dire [49]. Other

bacteria species such as *Burkholderia sp*. have also been reported to show resistance to monochloramine and chlorine in water [14].

Table 5.2. Identities of the dominant bacteria species in the wastewater sample

OTU	Bacteria strain*	Accession number	% Similarity
A	*Arcobacter suis F41*	NR _116729.1	99
B	*Pseudomonas sp strain QBA5*	MF782453.1	98
C	*Pseudomonas sp B-AS-44*	JF901706.1	99
D	*Pseudomonas sp CCI2E*	KM187145.1	100

*Based on the BLAST comparison of sequences to the NCBI database

Variations that exist in the responses of bacteria to diverse disinfectants or antiseptics are largely due to the differences in cellular structure, composition, and physiology. Bacteria susceptibility to disinfectants could be associated with a chromosomally controlled natural property of the organism (the intrinsic factor) or emanating from the genetic changes which develop from the acquisition of plasmids or transposons or by mutation (the acquired factor) [50-51]. Usually, an applied disinfectant inactivates bacteria by an initial interaction with the cell surface and subsequent penetration into the cell to reach its intracellular target sites.

The cell outer surface membrane thus plays a significant role in determining the viability or susceptibility to a disinfecting agent. For instance, the differences in the lipopolysaccharides (LPS) composition and the cation content of the outer membranes of *Pseudomonas aeruginosa* account for its high resistance to several antimicrobial agents [51]. Besides this, Gram-negative bacteria are generally less susceptible to disinfectants than Gram–positive [52] and this might partly explain why all the dominant species that survived the chlorine dioxide treatment and could be detected in the gel in this study are all Gram-negative- bacteria.

It is observed in this study that for a complex bacterial community in a wastewater system, the DGGE based culture-independent technique could be used for simultaneously determining the

responses of different species of bacteria to an applied disinfectant dose and indicating potential resistant species including the VBNC strains which could not be determined by the classical heterotrophic plate count technique. However, it is limited in depicting quantitatively, the log reductions of bacterial populations at the different concentrations of disinfectant

5.4 Conclusion

Denaturing Gradient Gel Electrophoresis (DGGE) has been demonstrated as a useful culture-independent technique for assessing the susceptibilities of diverse bacteria populations in a municipal wastewater to chlorine dioxide. It has also been established that chlorine dioxide is an effective disinfectant for controlling autochthonous bacteria during wastewater disinfection. However, in determining suitable doses of a disinfectant required for completely inactivating bacteria, marked differences might exist between the susceptibility data obtained from culture-based techniques and culture-independent methods such as DGGE. Consequently, the application of culture-dependent methods alone to monitor the efficiency of a water disinfection system might not be sufficient since considerable details of interest about other non-culturable bacterial strains with different susceptibilities to a disinfectant may not be known. It is envisaged that the results of this study will broadly influence the monitoring strategies for verifying the efficiency of a disinfection regime and prospecting for potential organisms developing resistance towards water disinfectants.

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CHAPTER 6

Ozone Initiated Inactivation of *Escherichia coli* and *Staphylococcus aureus* in water: Influence of Selected Organic Solvents Prevalent in Wastewaters.

Abstract

Absorption, stability and reactivity of ozone in water are critical parameters to determine its efficiency in microbial inactivation. In this study, the influence of four water-soluble organic solvents commonly discharged from industrial lines into wastewater systems, namely; ethanol, methanol, ethyl acetate and dimethyl sulfoxide (DMSO) on the ozone-facilitated inactivation of *Escherichia coli* and *Staphylococcus aureus* in water was investigated. Ozone absorption (up to 12 min) as function of ozone aeration time, and the decomposition rates were spectrophotometrically monitored in the presence of 2.5% and 5% concentrations of each organic solvent. Their consequent effect on bacterial inactivation was determined. The inactivation kinetics were described using the efficiency factor Hom model. Residual concentrations of absorbed ozone in solutions with ethyl acetate or DMSO were relatively higher than those in methanol or ethanol-containing solutions. DMSO and ethyl acetate enhanced the stability of ozone in water, characterised by a lower decomposition rate constant in DMSO (k_d = 3.81 x 10^{-2} M^{-1} s^{-1}) and ethyl acetate (k_d = 4.45 x 10^{-2} M^{-1} s^{-1}) solutions, in contrast with that in methanol (k_d = 1.13 x 10^{-1} M^{-1} s^{-1}), where the decomposition rate was higher. The faster absorption and stability of ozone in ethyl acetate and DMSO corresponded with an observed increase in the log inactivation of *E. coli* and *S. aureus* by approximately 2-fold relative to that in methanol.

Key words: Ozone stability; Organic solvents; Disinfection; *Escherichia coli*; *Staphylococcus aureus*

6.1 Introduction

In the last decade, various applications of water treatment, including pathogen control and oxidative degradation of inorganic and organic compounds, with its concomitant removal of odour, colour, and particles have well explored the strong oxidative properties of ozone. As a disinfectant in water, ozone is an excellent treatment option for achieving remarkable inactivation for viruses, *Giardia* and *Cryptosporidium*, making it suitable for meeting regulatory requirements [1]. Nevertheless, a major limitation is its short lifetime and hence its inability to provide residual quantities for downstream microbial inactivation activities. Consequently, in many water treatment processes, ozone is not employed as a final disinfectant.[2-3].

In recent times, there has been renewed interest in ozone utilisation for both drinking water and wastewater treatments worldwide. This is particularly due to advances in efficient and cost-effective ozone generation technologies, as well as the excellent ability of ozone to oxidise emerging micro-pollutants such as endocrine disrupting compounds and pharmaceutically active and personal care products to biodegradable levels [4-5]. More to this is the increasing regulations on disinfection byproducts, such as trihalomethanes, haloacetic acids and haloacetonitriles associated with conventional chlorination methods [6-7].

The reactions of ozone in water broadly proceed along two major pathways. Molecular ozone (O_3) may react directly with dissolved substances or undergo decomposition to produce secondary oxidants such as hydroxyl radicals (˙OH), a strong oxidant (E^o = 2.80 V), which react rapidly and non-selectively with solutes [8-9]. ˙OH radicals are also critical intermediates for promoting the radical chain decomposition reactions of ozone in water. However, in the presence of ˙OH radical scavengers such as tert-butanol, carbonates and acetates in water, ˙OH radicals are significantly quenched to enhance ozone stability. Several organic solvents including acetone, dimethyl sulfoxide (DMSO), 2-propanol, oxolane, 1,4-dioxane, methanol and ethanol have also been reported to react rapidly with ·OH radicals to the extent of scavenging approximately 70 - 90% thereof [10]. Compounds such as free amines, electron-rich aromatics and unsaturated hydrocarbons are more prone to quick ozone attack, whilst others including saturated hydrocarbons, alcohols, aldehydes and other organic and inorganic

contaminants, which are best considered as being resistant to ozone oxidation, react quickly with ˙OH radicals [11-12].

Disinfection of microbial contaminants in water is predominantly executed by the O_3 molecule instead of ˙OH radicals [13-14]. However, ozone is generally unstable in water [15]. Therefore, at any applied dose, the efficiency of ozone in micro-pollutant abatement and microbial inactivation is considerably a function of its stability and the degree of reactivity with the pollutants, which in effect tend to extensively influence the decomposition rate of ozone [16]. In drinking water and wastewater treatments, the extent of reactivity and stability of the applied ozone has been suggested to be largely dependent on the nature of compounds; particularly the dissolved organic matter in the water [12].

Organic solvents are contaminants of concern in water bodies and are usually discharged through various industrial activities [17-18]. The production processes in the pharmaceutical, cosmetic, textile, rubber, wine and other industries generate significant amounts of organic solvents as effluents released into wastewaters [19]. For instance, ethanol constitutes about 80 - 90% of the total chemical oxygen demand content in effluents of wine industries with reported values ranging from 5000 to 25000 mg/L [20-21].

Methanol and ethanol are also commonly utilised as external carbon sources for facultative heterotrophs involved in the removal of anoxic nitrogen during wastewater denitrification [22-23] and nearly 200 of such wastewater treatment plants operate in the US alone. DMSO, a common laboratory organic solvent which is also widely employed as a detergent and solvent for washing and rinsing in the semiconductor manufacturing industry is discharged in substantial quantities into wastewater from industrial production lines [24]. In some cases, DMSO levels exceeding 2400 mg/L were reported to have been discharged from an industrial source to a sewage collection system [25], whilst about 500 – 800 mg/L had also been found in effluents from a film transistor liquid crystal display wastewater [26]. Others such as ethyl acetate, used in the petrochemical and polymer industries and for cleaning paints are also prevalent in environmental waters [27].

Ozone reactivity towards some organic solvents such as tetrachloromethane, hexane, acetic acid, acetone, methyl acetate, and polydimethylsiloxanes is limited ; however, its solubility and stability in organic solvents are higher than those in pure water under similar conditions of temperature and ozone concentration [28]. This characteristic has been reported to increase the rate of ozone degradation of some hazardous organic compounds in wastewater [29]. Therefore, the presence of certain organic solvents in water could enhance ozone stability and solubility, which are important factors that influence its disinfection efficiency. Furthermore, the competitive kinetic reactions between ozone and dissolved organic solvents could also possibly shield microorganisms from the oxidative power of the applied ozone, and thereby result in reduced disinfection efficiency.

In the present study, we focused on determining the influence of the presence of methanol, ethanol, DMSO, and ethyl acetate as model organic solvents that are commonly found in wastewater matrices on the efficiency of ozone inactivation of *Escherichia coli* and *Staphylococcus aureus*. *E. coli* is a Gram-negative bacterium and a common species of fecal coliforms [30] found in the intestinal tracts of warm-blooded animals. It is prevalent in natural waters and wastewaters and commonly used as an indicator organism to assess the efficiency of water disinfection [31]. *S. aureus* is a nonsporulating Gram-positive bacterium, which is relatively widespread in the environment but frequently found on the mucous membranes and skins of animals. It is generally transferred into water by human contact in swimming pools, spa pools, and other recreational waters and has been cited as the leading cause of foodborne infections [32]. The methicillin-resistant *S. aureus* strains have particularly gained attention in recent times due to the potential risk of transmitting infections from animals to humans [33]. The contributions of individual anti-microbial activities of the selected organic solvents to the overall disinfection efficiency in the water, and to the ozone decomposition kinetics are considered.

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6.2 Materials and Methods

All chemicals and reagents used in the study were of analytical grade (≥ 95% purity) and purchased from various commercial suppliers and used as received. These included methanol, ethanol and DMSO (Sigma Aldrich) as well as ethyl acetate (BDH Analytical Chemicals, UK). Solutions and media were prepared with Millipore water.

6.2.2 Bacterial strains and preparation.

The *E. coli* (ATCC 25218) and *S. aureus* (ATCC 29213) strains were obtained from the Microbiology Discipline, University of KwaZulu-Natal, Westville. Stock culture samples were grown overnight in a 100 mL 1X lysogeny broth at 37 ± 2 ºC, harvested by centrifugation, washed twice in 0.1 M phosphate buffered saline (pH 6.8) and resuspended in fresh portions of the same buffer.

6.2.3 Ozone generation, calibration, and measurement

Oxygen-rich ozone gas was generated by the corona discharge method from a Mighty Zone ozoniser (mzpvL-8000, Powertron Electronics, South Africa) using high grade compressed oxygen gas as the generation source. Before conducting the disinfection studies, the ozoniser was calibrated to determine the concentrations of absorbed ozone at 2.5% and 5% doses (v/v) of each solvent at a temperature of 22 ± 2 ºC in phosphate buffered laboratory water. In this process, the ozone generation and flow were stabilised by initially bubbling the output ozone continuously for approximately 10 min at a feed-gas flow rate of 2.0 L/min, adjusted using a mass flow meter, directly into an ozone destruct unit made up of a glass bottle containing 300 mL of 2% potassium iodide (KI) solution. Subsequently, the oxygen-rich ozone gas was fed into the reactor containing 200 mL of the test solution by bubbling through a fritted glass gas diffuser at an ozone product-gas flow rate of 30 mL/min whilst maintaining the output conversion efficiency at 20%. This was repeated for different predetermined time periods (2, 4, 6, 8 10 and 12 min), and in each case, the ozone concentration was measured. The time-course absorption of ozone approach under similar experimental conditions was used to determine the influence of each solvent on the rate of ozone dissolution or absorption when bubbled into the water. The ozone concentration was determined spectrophotometrically and

confirmed by the iodometric method, involving the oxidation of 2% KI solution to liberate I_2, which was then titrated with standardised sodium thiosulphate [34].

6.2.4 Ozone aeration set-up and disinfection procedure

Preliminary experiments were conducted to investigate the antimicrobial activity of each organic solvent on the studied organisms at 1%, 2.5%, 5% and 10% doses (v/v), but without ozone bubbling. However, in practice, the doses employed in industrial wastewaters are quite diverse, for instance; ethanol (25000 mg/L ≈ 3.6% v/v), DMSO (2400 mg/L ≈ 0.2% v/v), and methanol (1000 mg/L ≈ 0.12% v/v) [20, 25, 35]. Cell suspensions of each bacterium at ~10^8 cfu/mL were incubated with solvents for 1 h at room temperature (22 ± 2) ºC under aseptic conditions; subsequently, the samples were serially diluted and plated on nutrient agar to enumerate the surviving cells. Considering the susceptibilities of the organisms to the solvents (section 6.3) and for the purposes of our study, further bacterial inactivation experiments were conducted at 2.5% and 5% (v/v) solvent concentrations in water.

A schematic diagram of the set-up for ozone disinfection of bacteria in solutions of the studied organic solvents is shown in Figure 6.1. The reactor consisted of a 500 mL glass bottle fitted with an inlet for bubbling ozone gas and an outlet for the passage of excess ozone into the destruct unit containing KI solution. An additional port fixed with a septum allowed for the injection of bacteria samples into the reactor and for sampling at a given time point using a 10 mL sterile syringe. Ozone was bubbled into a 200 mL phosphate buffered (pH 6.8) test solution (2.5% or 5% organic solvent) in the reactor at a feed gas flow rate of 2.0 L/min for 4 and 12 min on the basis of the predetermined calibrations. The prepared bacteria suspensions were immediately transferred into the reactor at an approximate density of 1 – 2 x 10^8 cfu/mL. The control disinfection experiments involved similar treatments of the cells with ozone under identical conditions but without any organic solvent. Unless otherwise stated, all the experiments were conducted independently in triplicate, at 22 ± 2 ºC under agitation using a magnetic stirrer at 100 rpm to create a uniform distribution of ozone and bacteria whilst minimising desorption. After initiating each disinfection reaction, 5.0 mL portions were sampled from the reactor at predetermined time intervals and transferred into 10 mL tubes containing 2.0 mL of 0.1 M $Na_2S_2O_3.5H_2O$, to quench the ozone reaction. Enumeration of surviving bacteria was determined by the heterotrophic plate count method on a nutrient agar incubated overnight at 37 ºC.

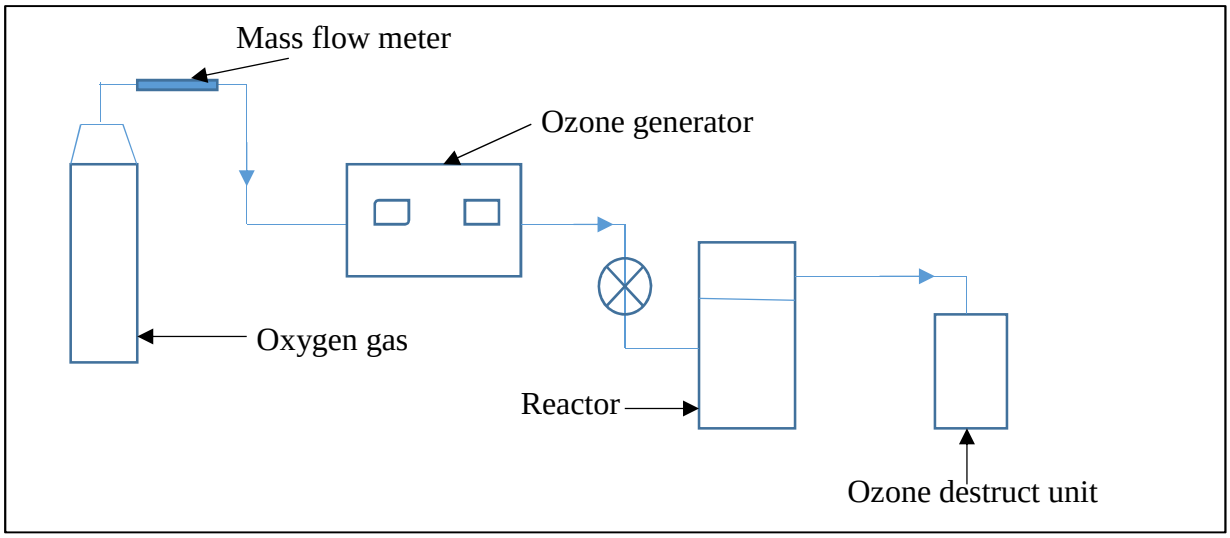

Figure 6.1 A schematic diagram of the set–up used for the ozonation of bacteria

6.2.5 Decomposition kinetics of ozone in the presence of organic solvents.

To determine the stability of ozone in water in the presence of each organic solvent, the decay kinetics of ozone were assessed. Ozone gas was bubbled into 200 mL of double distilled water (Millipore) at an output flow rate of 2 L/min for 1 h to obtain a saturated stock solution of approximately 15 mg/L. Subsequently, a calculated amount of the stock solution was transferred into the reactor containing a test solution of the organic solvent as described above to obtain a final desired concentration of 3.0 mg/L. Samples were monitored to determine the rate of decay by measuring the changes in absorbance at 260 nm as a function of time in a 1.0 cm quartz cuvette using a UV-visible spectrophotometer (Libra S12, Biochrom, Cambridge, England). Ozone concentrations were determined at a molar absorption coefficient, ε of 3200 M^{-1} s^{-1} [12]. Control experiments were similarly conducted but without the organic solvents. At higher pH conditions, and most especially in basic solutions, the decay rate of ozone is well known to be significantly accelerated by hydroxyl ions [12, 36]. However, at pH below 7, it does not decay appreciably and hence present as the O_3 molecule [37]. Therefore, to minimise

interferences by OH^- ions and for the purposes of this study, all decomposition experiments were conducted in a phosphate buffered water at an adjusted pH of 6.8 at 22 ± 2 ºC.

6.2.6 Data and statistical analysis

On the basis of the assumption that the rate of ozone decomposition in terms of the direct reaction with dissolved substances, [M], follows *pseudo* first-order kinetics with respect to $[O_3]$ [38-39], the rate law could be expressed as follows:

$$\frac{d[O_3]}{dt} = k_d[O_3][M]^n \quad (1)$$

where k_d is the rate constant for the decomposition of ozone. When [M] is present in excess ($[O_3]_o$ <<< [M]) and thus assumed to be constant, the integral of equation 1 yields the *pseudo*-first order reaction rate as follows:

$$\ln\left(\frac{[O_3]t}{[O_3]o}\right) = k_d[M]^n.t \quad (2)$$

where $[O_3]_o$ and $[O_3]_t$ are concentrations of ozone at time t = 0 and time t respectively. The rate constants for ozone decomposition k_d (s^{-1}) in the aqueous solutions of the solvents were therefore determined from equation 2.

The kinetics of bacterial inactivation with ozone in different organic solvent conditions were analysed by the efficiency factor Hom model [40-41] as shown in equation 3. This model accounts for the ozone disappearance rate constant and best fitting the tailing–off behaviour of the inactivation curves.

$$\mathbf{Log}\left(\frac{N_t}{N_O}\right) = \boldsymbol{k}\mathbf{C_o^n T^m}\,\boldsymbol{\eta} \quad (3)$$

$$\boldsymbol{\eta} = \left[\frac{1-exp(-nk_dT)}{nk_dT/_m}\right]^{\mathbf{m}}$$

where $\mathbf{(N_t/N_o)}$ is the survival ratio of the microorganism under consideration after the inactivation, $\boldsymbol{k}$ is the microbial inactivation rate constant, $\mathbf{C_o}$ is the initial residual concentration of the disinfectant in water, **T** is the contact time of exposing the disinfectant to the microorganism, **m** is a model parameter called the Hom exponent and **n** is the *coefficient of dilution*. The dimensionless efficiency factor $\boldsymbol{\eta}$ accounts for the loss of the disinfectant residual during the contact time. Model parameters were determined by minimising the error sum of squared deviations between the observed and predicted log (N_t/N_o) of the inactivation data using Microsoft Excel Solver (Microsoft Corp, 2016). The student *t*-test based on *p*-value at

95% confidence level was used to evaluate the statistical significance of data from at least triplicate independent measurements.

6.3 Results and Discussion

6.3.1 Dissolution of ozone in water in the presence of organic solvents

The dissolution of ozone in water in the presence of the chosen organic solvents for the study, namely ethanol, methanol, DMSO and ethyl acetate is as indicated in Figure 6.2. Ozone was bubbled into the water for 2 , 4 , 6 , 8 , 10 and 12 min durations, and the dissolved concentrations in each solvent increased with the ozone aeration time. However, at the same flow rate and with all physical parameters kept constant, the differences in the ozone concentrations in terms of different solvent concentrations (2.5% or 5%) were found to be statistically insignificant ($p > 0.05$). Relatively higher residual ozone concentrations were obtained in the solutions containing ethyl acetate, DMSO or ethanol than in the control and methanol under comparable conditions. For instance, after a 12 min continuous bubbling of ozone-enriched oxygen in 5% solvent-containing water, the residual concentrations (mg/L) obtained were 0.98 ± 0.062, 0.85 ± 0.088, 0.59 ± 0.031, 0.90 ± 0.065 and 0.64 ± 0.043 for ethyl acetate, ethanol, methanol, DMSO and the control respectively.

Ozone is a sparingly soluble gas, and its dissolution or absorption in water is critical for ensuring its efficiency in disinfection or micropollutant oxidation [12]. The transfer of ozone into water is simultaneously controlled by the efficiency of the mass transfer process and the irreversible chemical reactions that occur within the system [2]. Mass transfer efficiency is influenced largely by physical parameters such as the temperature, gas flow rate, partial pressure, hydrodynamics (i.e., gas-liquid phase mixing) and diffuser type [42] among other factors. Moreover, the concentration and nature of the dissolved organic or inorganic moieties in the water are significant determinants of the transfer and reaction rates and the consequent residual of ozone for disinfection in water. In some instances, samples that contain fast ozone reacting species such as Fe^{2+}, Mn^{2+}, SO_3^{2-},NO_2^- and phenol, the transfer process may proceed in such a manner that limited or no ozone residual will be left at the end of the process [43-44].

In our present study, where all the physical parameters were kept constant in each experimental trial, the different concentrations of absorbed ozone as a function of bubbling time likely

resulted from the chemical interactions of the dissolved organic solvents with ozone. Generally, the consumption of molecular ozone by direct reactions with most organic solvents is slow with a rate constant k of $< 10^3\ M^{-1}\ s^{-1}$ [38]; however, other mechanisms facilitated by $^{\cdot}OH$ radicals could accelerate the decomposition rate. For instance, a relatively higher decomposition rate of ozone in methanol by free radical-mediated reactions (section 6.3.2) could possibly account for the relatively lower residual concentrations obtained.

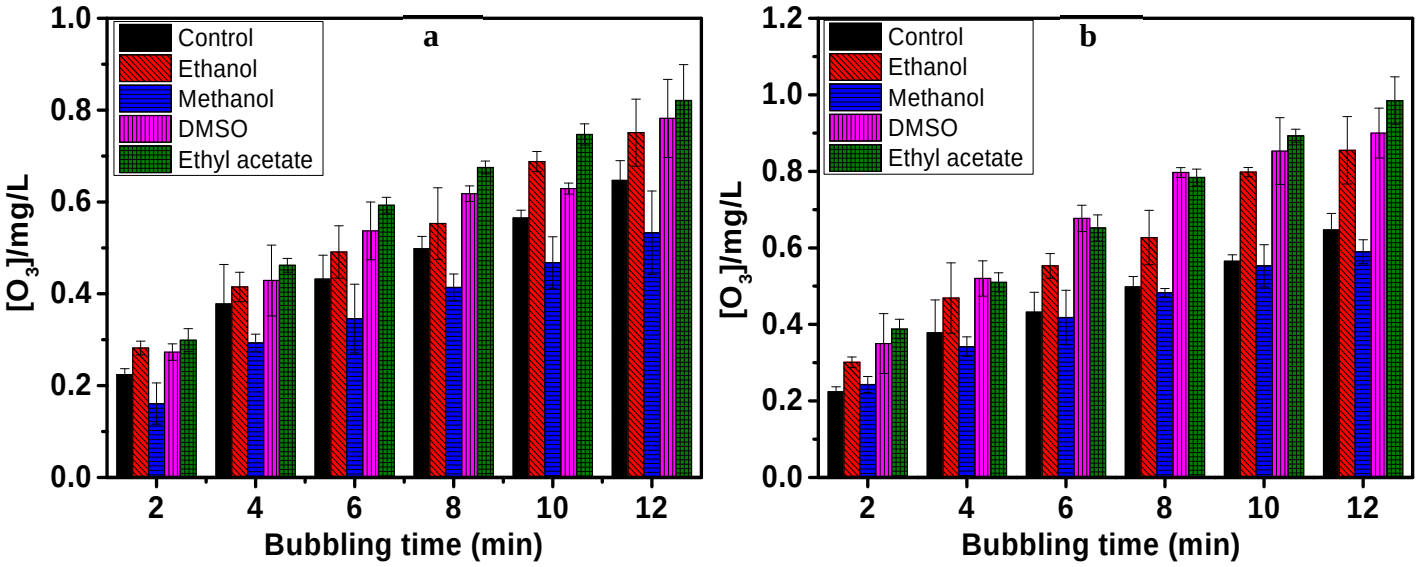

Figure 6.2. Residual concentrations of ozone in water as a function of bubbling time at pH 6.8, 22 ± 2 ºC and flow rate (2.0 L/min) in the presence of (a) 2.5 % and (b) 5.0 % of organic solvents

6.3.2 Ozone decomposition kinetics in the presence of organic solvents in water

In this study, we sought to determine the rate of ozone depletion in water containing low concentrations of selected organic solvents. Plots of the decomposition kinetics are illustrated in Figure 6.3, whilst Table 6.1 shows a summary of the decomposition rate constants as determined from equation 2. The overall decay rate constants indicate a relatively faster ozone

decomposition in methanol and ethanol solutions than that in ethyl acetate and DMSO. At 5% (v/v) concentrations of the solvents, the decay constant k_d for methanol was 1.13 x 10^{-1} M^{-1} s^{-1}, whilst that in ethanol under similar experimental conditions was 7.94 x 10^{-2} M^{-1} s^{-1}. However, the decomposition rates of ozone in ethyl acetate and DMSO solutions were also significantly lower, than those in control experiments, which contained only phosphate-buffered solutions. This suggests improved ozone stability in the presence of these solvents. For each of the studied organic solvents, the differences in the rate of decay between the two concentrations (2.5% and 5%) as determined by the decay constants were not statistically significant ($p > 0.05$). Once bubbled into the water, ozone undergoes continuous dissipation during the entire contact time owing to either volatilisation, auto-decomposition and/or reaction with the water constituents [43]. The ozone decomposition process in natural waters or wastewaters reportedly contains two phases. The first phase of the decomposition process occurs before the initial 20 s, where very fast reacting species rapidly or almost instantaneously consume ozone and this is operationally termed as the instantaneous ozone demand (IOD) [45]. It is followed by a second phase, which empirically follows a first-order kinetic rate law [39, 45] and whose kinetic rate constant is largely dependent on the constituents of the water.

The decomposition process involves radical chain reactions initiated predominantly by hydroxyl ions OH^- to form other secondary oxidants such as $^{\cdot}OH$ radicals, superoxide anion ($O_2^{\cdot -}$) and hydroperoxyl radicals ($HO_2^{\cdot}$) to further accelerate the reaction as indicated in equations (4 – 11) [36, 46-47]. This process is therefore catalysed by an increase in pH due to the increased OH^- concentration.

$$\mathbf{O_3 + OH^- \rightarrow HO_2^{\cdot} + O_2^{\cdot -}}$$ Initiation step **(4)**

$$\mathbf{HO_2^{\cdot} \leftrightarrow O_2^{\cdot -} + H^+}$$ **(5)**

$$\mathbf{O_3 + O_2^{\cdot -} \rightarrow O_3^{\cdot -} + O_2}$$ key propagation step **(6)**

$$\mathbf{O_3^{\cdot -} + H^+ \rightarrow HO_3^{\cdot}}$$ **(7)**

$$\mathbf{HO_3^{\cdot} \rightarrow HO^{\cdot} + O_2}$$ **(8)**

$$\mathbf{O_3 + HO^{\cdot} \rightarrow HO_4^{\cdot}}$$ **(9)**

$$\mathbf{HO_4^{\cdot} \rightarrow HO_2^{\cdot} + O_2}$$ **(10)**

$$\mathbf{HO_4^{\cdot} + HO_4^{\cdot} \rightarrow H_2O_2 + O_2}$$ Termination step **(11)**

However, in a complex water system such as natural waters or wastewaters, the various dissolved solutes could alternatively initiate, promote or inhibit the radical chain decomposition reactions on the basis of the capacity to convert ·OH to $O_2^{\cdot -}$ [36]. Hoigne and Bader reported a slow direct reaction kinetics between ozone and methanol or ethanol with second order rate constants of approximately 0.024 and 0.37 M^{-1} s^{-1} respectively at pH 2. Ozone depletion in the ethanol-containing water was faster than that in methanol, at low pH, and where a large excess of propanol or tert-butanol had been added to scavenge ·OH radicals to eliminate any influences arising from free radical reactions [46]. However, under the current study conditions, the kinetics of ozone depletion were faster in methanol-containing water than in ethanol-containing water.

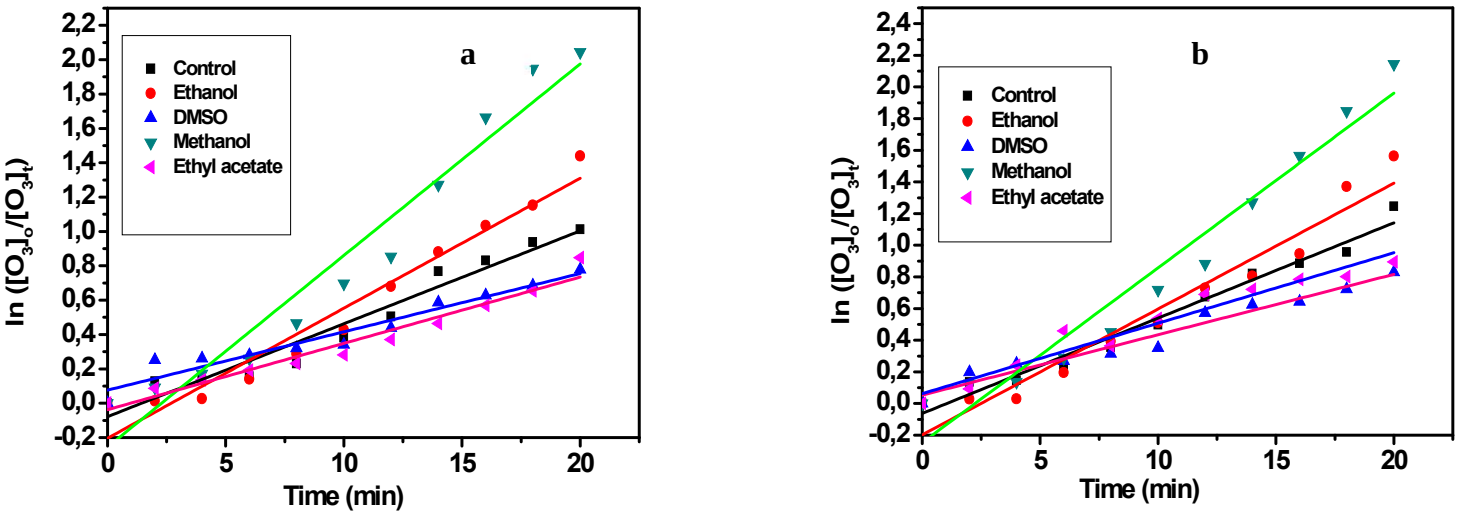

Figure 6.3 Ozone decomposition kinetics in water in the presence of (a) 2.5% and (b) 5.0 % of organic solvents determined at pH 6.8 and 22 ± 2 ºC

When ozone-enriched oxygen is bubbled into water in the presence of methanol, a series of radical reactions occur. Hydrogen atoms, α to the alcoholic (-OH) group are abstracted to generate $O_2^{\cdot -}$ radical, which is a key free radical required for the propagation of the radical chain reaction mechanism of ozone decomposition as illustrated in equations (12 – 14) [48].

Methanol is therefore regarded as a promoter of ozone decomposition. On the contrary, in the case of ethanol, ˙OH radicals may abstract such non α-H atoms but do not yield the superoxide radicals that are required to accelerate the decomposition reaction [36]. In such circumstances, ˙OH radicals are scavenged and eventually inhibit ozone decomposition. This might account for the relatively faster ozone depletion in methanol than in ethanol. DMSO also undergoes slow direct reaction with ozone ($k \approx 1.8\ M^{-1}s^{-1}$), but rapidly reacts with ˙OH ($k \approx 7 \times 10^9\ M^{-1}\ s^{-1}$)[12].

This hydroxyl radical scavenging characteristic potentially curtails the radical decomposition reaction of molecular ozone, thus enhancing its stability and resulting in a lower decomposition rate constant. The slow direct reaction and lower decomposition rate may also account for the observed relatively higher residual concentration of bubbled ozone in both ethyl acetate and DMSO solutions in water. As described by Rabani et al., [48].

$$\mathbf{CH_3OH + O_3 \rightarrow {}^{\cdot}CH_2OH + HO_3^{\cdot}} \qquad \mathbf{(12}$$

$$\mathbf{{}^{\cdot}CH_2OH + O_2 \rightarrow {}^{\cdot}OOCH_2OH} \qquad \mathbf{(13)}$$

$$\mathbf{\cdot OOCH_2OH + OH^- \rightarrow CH_2O + H_2O + O_2^{\cdot -}} \qquad \mathbf{(14)}$$

Table 6.1. Rate constants of ozone decomposition in aqueous solutions of organic solvents determined at pH 6.8 and 22 ± 2 ºC

Solvent	**% v/v**	**Initial $[O_3]$/mg/L**	**k_d (M^{-1} s^{-1})**	**R^2**
Control (Phosphate Buffered water)	-	3.0	0.0602	0.97
Ethanol	2.5	3.0	0.0757	0.93
	5.0	3.0	0.0794	0.95
Methanol	2.5	3.0	0.1110	0.94
	5.0	3.0	0.1130	0.95
DMSO	2.5	3.0	0.0339	0.95
	5.0	3.0	0.0381	0.94
Ethyl acetate	**2.5**	**3.0**	**0.0386**	**0.95**
	5.0	**3.0**	**0.0445**	**0.94**

6.3.3 Ozone disinfection of bacteria in the presence of organic solvents

The bactericidal activities of the organic solvents at the doses considered in this study in the absence of ozone are summarised in Table 6.2. Less than 1-log inactivation was obtained for both *E. coli* and *S. aureus* at solvent concentrations of 5% or less in water. However, at the 10% dose, ethanol was observed to kill up to 7 and 6 logs of *E. coli* and *S. aureus* respectively whilst methanol inactivated nearly 1-log of each bacterium. DMSO and ethyl acetate were virtually inactive at all the concentrations considered, suggesting that their presence in environmental wastewater may not contribute significantly to the overall bactericidal activity.

Ethanol is a common disinfectant or antiseptic agent that exhibits broad-spectrum activity against viruses, bacteria, and fungi [49] and is applied to skin surfaces, medical instruments and other hard surfaces in hospitals to control nosocomial infections [50]. Absolute ethanol is generally ineffective, as efficient microbiocidal activity of ethanol requires the presence of water, to rapidly denature proteins and consequently interfere with cell metabolism. Hence, optimal biocidal activity is usually at 60 – 70% ethanol concentrations, but this is significantly reduced at dilutions lower than 50% [51]. However, in our present study in water, ethanol was effectively bactericidal at concentrations as low as 10%. In contrast, methanol was observed to poorly inhibit bacterial growth.

Table 6.2 Inactivation of *E. coli* and *S. aureus* by organic solvents incubated for 60 min in the absence of ozone at 22 ± 2 ºC

	% Solvent concentration in water			
	1.0	2.5	5.0	10.0
Log (N_t/N_o)				
E. coli				
Solvent				
Control	< 0.10	< 0.10	< 0.10	< 0.10
Ethanol	< 0.10	0.45	0.76	7.21
Methanol	< 0.10	0.32	0.55	1.20
DMSO	< 0.10	< 0.10	< 0.10	0.22
Ethyl acetate	< 0.10	< 0.10	< 0.10	0.18
S. aureus				
Control	< 0.10	< 0.10	< 0.10	< 0.10
Ethanol	< 0.10	0.24	0.65	6.14
Methanol	< 0.10	0.19	0.43	0.89
DMSO	< 0.10	< 0.10	< 0.10	0.15
Ethyl acetate	< 0.10	< 0.10	< 0.10	0.11

Figure 6.4 shows the inactivation profiles of *E. coli* and *S. aureus* after bubbling ozone for 12 min under the experimental conditions in the presence of 2.5% and 5% organic solvents. The estimated parameters of the fitted efficiency factor Hom model (equation 3) from the inactivation data are shown in the supporting information Tables S6.1 and S6.2 (pages 146 and 147) The results of the extent of inactivation observed when ozone was bubbled for 4 min are also summarised in the supporting information, (Figure S6.1, page 148). In ethyl acetate (2.5 or 5%), very effective inactivation, i.e., approximately 6-log reduction of *E. coli* in a contact time of 6 min, was found, whilst in the presence of ethanol and DMSO, at least 5-log reduction

was obtained. Furthermore, under similar conditions, ~ 3-log reduction was achieved in the case of methanol and the control. Similar results were observed for Gram-positive strain, *S. aureus*. Deductions from the inactivation data of the individual organic solvents (Table 6.2) suggest that the differences in the degree and rate of inactivation are largely a function of the residual concentrations of the absorbed ozone per unit time as discussed above. This observation is due to the effect of marginal differences in absorbed ozone on the overall inactivation efficiency. However, these differences could be eliminated, when ozone is bubbled for a longer time to ensure that all possible initial demands are satisfied. Relatively, the faster absorption rate of ozone per unit time and the enhanced stability of ozone in ethyl acetate and DMSO, leading to the maintenance of high concentrations of dissolved ozone in contact with the bacteria may be responsible for the higher inactivation.

Apart from the disinfection of bacteria, it may also be expected that the solubility and stability of aerated ozone in wastewater systems containing organic solvents such as ethyl acetate and DMSO will improve the oxidation of other pollutants that react directly with molecular ozone in water. Further, the consumption of ˙OH radicals produced from the side reactions of ozone with the effluent organic matter may also limit the oxidation of species that are indirectly oxidised by ozone through the ˙OH radical route.

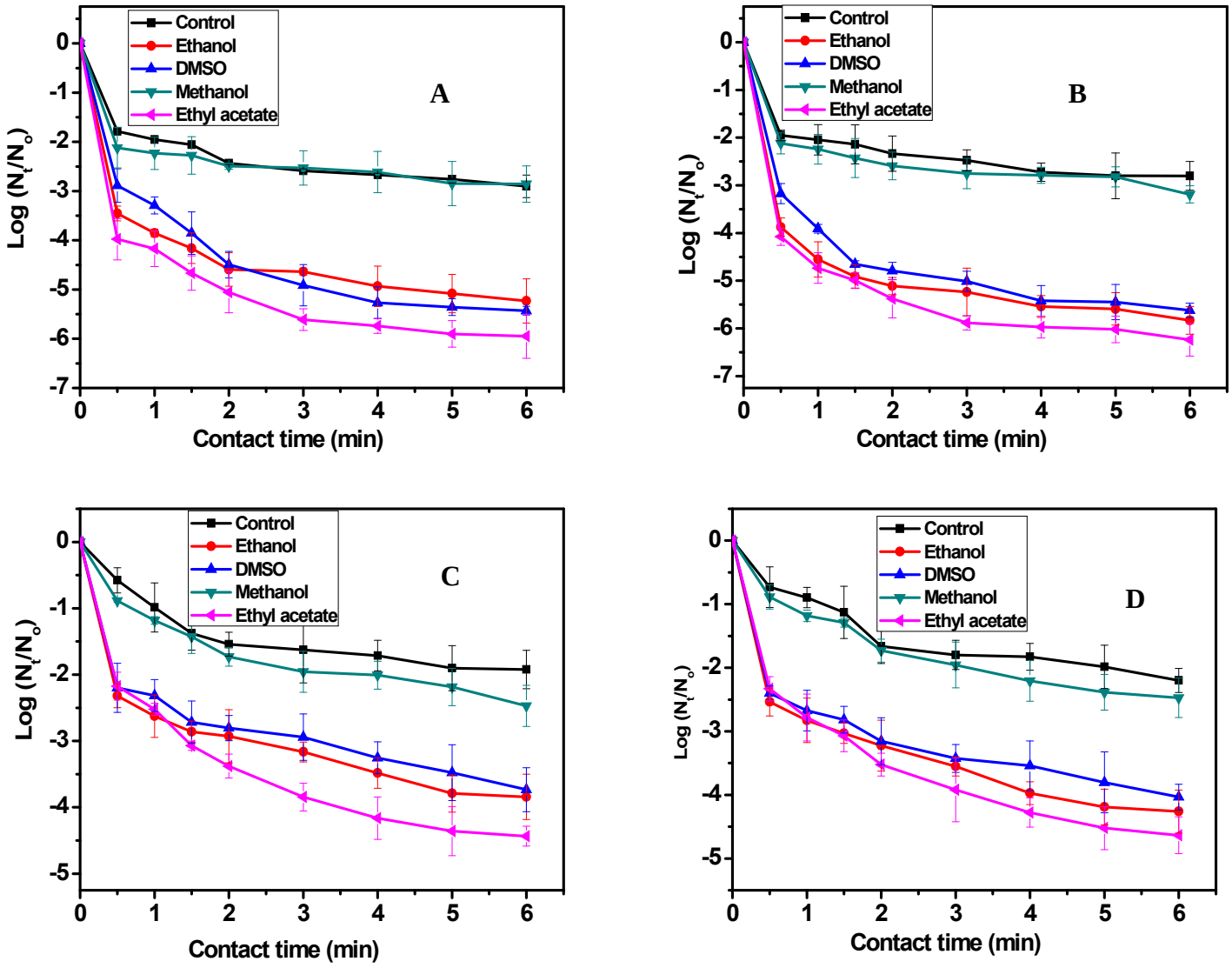

Figure 6.4. Bacteria inactivation profiles with ozone bubbled for 12 min at pH 6.8 and 22 ± 2 ºC for (A) *E. coli* in 2.5% organic solvent (B) *E. coli* in 5% organic solvent (C) *S. aureus* in 2.5% of organic solvent and (D) *S. aureus* in 5% of organic solvent.

6.4 Conclusion

The study demonstrated that water-soluble organic solvents such as ethyl acetate and DMSO at concentrations up to 5% could significantly enhance the rate of ozone absorption and decrease the rate of ozone decomposition. Hence, the stability and consequently the bacterial inactivation efficiency of applied ozone are enhanced. However, in the presence of methanol, the rate of ozone absorption is relatively reduced, possibly due to the simultaneous increase in the decomposition rate of the transferred ozone, which in turn, reduces the disinfection efficiency. At 5% concentrations or below, ethanol, methanol, DMSO and ethyl acetate do not independently contribute significantly to the overall bactericidal activity in water treatment. The enhanced solubility and stability of ozone in the presence of these organic solvents potentially enhance the micropollutant degradation efficiency of ozone during wastewater treatment.

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Supporting Information

Ozone Initiated Inactivation of Microbes, *Escherichia coli* and *Staphylococcus aureus* in water: Influence of Selected Organic Solvents Prevalent in Wastewaters.

Table S6.1 Estimated parameters for fitted Efficiency Hom model for ozone inactivation of *E. coli* in the presence of organic solvents determined at pH 6.8 and 22 ± 2 ºC

Solvent	**% v/v**	**Parameter**			
		k	**m**	**n**	**R^2**
Control (Phosphate Buffered water)	-	2.170	0.206	0.174	0.995
Ethanol	2.5	4.163	0.165	0.168	0.997
	5.0	4.104	0.165	0.167	0.996
Methanol	2.5	2.536	0.129	0.176	0.997
	5.0	2.466	0.129	0.184	0.993
DMSO	2.5	3.755	0.266	0.171	0.999
	5.0	3.693	0.266	0.168	0.998
Ethyl acetate	**2.5**	**4.571**	**0.182**	**0.167**	**0.998**
	5.0	**4.552**	**0.182**	**0.166**	**0.992**

Table S6.2. Estimated parameters of fitted efficiency Hom model for ozone inactivation of *S. aureus* in the presence of organic solvents determined at pH 6.8 and 22 ± 2 ºC

Solvent	**% v/v**	**Parameter**			
		k	**m**	**n**	**R^2**
Control (Phosphate Buffered water)	-	1.172	0.369	0.198	0.995
Ethanol	2.5	2.741	0.213	0.198	0.994
	5.0	2.753	0.213	0.183	0.996
Methanol	2.5	1.393	0.381	0.188	0.992
	5.0	1.401	0.382	0.182	0.993
DMSO	2.5	2.533	0.233	0.176	0.997
	5.0	2.594	0.233	0.181	0.996
Ethyl acetate	**2.5**	**2.814**	**0.298**	**0.178**	**0.992**
	5.0	**2.892**	**0.298**	**0.165**	**0.992**

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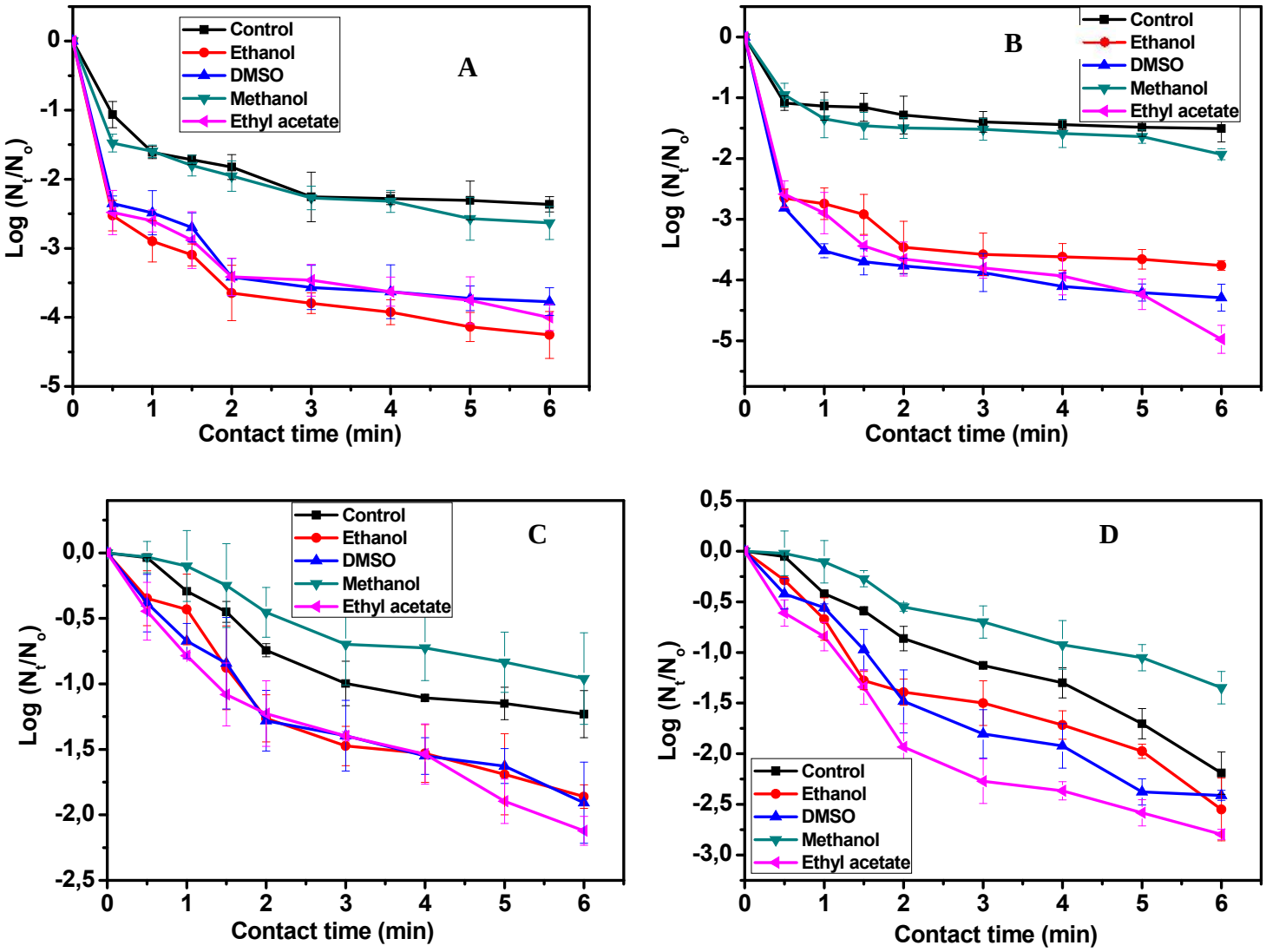

Figure S6.1 Bacteria inactivation profiles with ozone bubbled for 4 min at pH 6.8 and 22 $\pm 2^0$C for (A) *E. coli* in 2.5 % organic solvent (B) *E. coli* in 5.0 % organic solvent (C) *S. aureus* in 2.5 % of organic solvent and (D) *S. aureus* in 5.0 % of organic solvent

CHAPTER 7

CONCLUSIONS

In the present work, chlorine dioxide was studied as a disinfectant for the control of bacteria in water samples. The kinetics of its disinfection on three bacterial species: *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were monitored. The effects of parameters such as pH, temperature, and bacteria density and various dosage conditions were also assessed and characterised. In addition, the bactericidal mechanism of chlorine dioxide was investigated on all the test organisms. The efficiency of chlorine dioxide for controlling autochnous bacteria samples in wastewater was also evaluated by both culture-dependent heterotrophic plate count method and a culture-independent PCR-DGGE technique.

Ozone is another disinfectant that has been extensively studied. However, the absorption and stability in water are critical distinguishing factors of its application in water. The influence of some selected organic solvents prevalent in wastewater on its absorption and stability were explored and related to its efficiency at inactivating *E. coli* and *S. aureus*.

The key findings from this work are summarised in the section below

7.1 Summary of key findings

- Chlorine dioxide is an effective disinfectant for the control of bacteria in aqueous systems. At any given concentration, the reactions with bacteria were observed to be quite rapid and almost reaching its maximum inactivation at that concentration within the initial 2 min. Longer contact times did not yield any significant increase in the log reduction levels of inactivation achieved. This implies that using CT values in practice to predict the desired level of inactivation of bacteria in water may be unreliable with chlorine dioxide.
- Due to the production of disinfection by-products such as ClO_2^-, regulatory bodies like the WHO recommend that the maximum doses applied in drinking water should not exceed 1.5 mg/L. However, the findings of this work indicate that, at such concentrations, bacteria species like *P. aeruginosa* and *S. aureus* may not be effectively inactivated.

- The efficiency of chlorine dioxide as a disinfectant is optimal under alkaline pH conditions as compared to acidic or near neutral conditions in water. Higher doses may be required to obtain the desired level of inactivation of bacteria at lower pH. This is considered very significant in optimizing disinfection operations at a treatment plant when using chlorine dioxide.
- The rate of bacterial inactivation by chlorine dioxide was observed to be independent of the initial bacterial density present in water before disinfection.
- In general, increasing the temperature of the reaction system increases the rate of bacterial inactivation by chlorine dioxide. Raising temperatures from 4 °C to 15 °C produced substantial increases in the inactivation rate. However, increases from 15 °C to 37 °C yielded only marginal increases in the rate.
- Chlorine dioxide was also found to be effective at inactivating bacteria in wastewater. At a concentration of 3.0 mg/L, almost all heterotrophic bacteria contained in the urban wastewater samples studied were essentially eliminated.
- PCR based DGGE technique was successfully modified and utilised to assess the susceptibilities of diverse autochnous bacteria populations from wastewater samples exposed to varying concentrations of chlorine dioxide. It was found to be a valuable tool to monitor bacteria susceptibilities beyond the domain of culturable species and to prospect for potential resistant strains of bacteria to a disinfectant. However, some differences might exist between suitable doses required for complete inactivation when compared to culture-dependent methods like heterotrophic plate count.
- Chlorine dioxide was observed not to kill bacterial cells by inflicting gross morphological damage to cells or causing cell lysis. Instead, it was found to increase the permeability of the outer membrane, disrupting the integrity of the cytoplasmic membrane which forces the efflux of cytoplasmic contents such as DNA. This could lead to the loss of cell activity and consequently account for cell death or inactivation.
- The presence of ethyl acetate and DMSO in water were found to increase the absorption and stability of ozone in water which consequently improves its disinfection activity. On the contrary, methanol was found to accelerate the decomposition of ozone in water, thus reducing its residence time and adversely affecting its disinfection activity.
- At 5% concentrations or below, neither ethanol, methanol, DMSO nor ethyl acetate independently contribute significantly to the overall bactericidal activity in water treatment.

7.2 Recommendations for future research

The advantage of not forming highly regulated disinfection by-products has made chlorine dioxide a suitable alternative disinfectant for several applications. Increasingly, ClO_2 is used extensively in the food industry, in areas such as the decontamination of poultry processing water, meat and seafood products, sanitizing fresh fruits and vegetables and disinfecting surfaces of processing equipment. Moreover, it is now widely applied in hospital settings for wastewater disinfection and sanitizing equipment and even as a spray or fogging agent for plant pathogens control. These broad applications may have the potential of stimulating resistance among various microbial targets. Extensive research in this direction, aimed at characterising various genomes of microbial isolates to establish the existence of such possibilities is vital.

More work is also required on the by-product chemistry of ClO_2 application in the food industry and as an oxidant for emerging micropollutants in wastewater effluents, most especially on halogenated organic by-products. Similarly, more data on the fate of emerging pollutants such as pharmaceuticals and personal care products oxidised by O_3 in wastewater are needed to establish the background for appropriate guidelines regarding their application.

The design of suitable models for predicting the decay of ClO_2 and O_3 based on the water quality parameters is also necessary to enhancing their application and addressing some of the key challenges of safety and inadequate disinfection.

APPENDICES

Table A1. Data of ClO_2 disinfection of *E. coli* at varying concentration of oxidant

[ClO_2]/mg/L	**0.50 mg/L**		**1.00 mg/L**		**2.50 mg/L**		**3.50 mg/L**		**5.00 mg/L**	
Contact time(min)	**Log (Nt/No) ± SD**									
0	0		0		0		0		0	
0,5	-0,29	±0,031	-3,171	0,23	-5,55	±0,72	-5,954	±0,38	-7,161	±0,02
1	-0,41	±0,029	-3,787	0,31	-6,45	±0,54	-6,910	±0,45	-	-
2	-0,47	±0,017	-4,051	0,29	-6,47	±0,48	-	-	-	-
5	-0,49	±0,041	-4,285	0,34	-	-	-	-	-	-
30	-0,51	±0,026	-4,528	0,42	-	-	-	-	-	-
60	-0,54	±0,021	-4,732	0,39	-	-	-	-	-	-

Table A2. Datasheet of ClO_2 disinfection of P. aeruginosa at varying oxidant concentration

[ClO_2]/mg/L	**0.50**		**1.00**		**2.50**		**3.50**		**5.00**	
Contact time(min)	**Log (N_t/No) ± SD**									
0	0		0		0		0		0	
0,5	-0,114	±0,048	-1,905	±0,853	-2,495	±0,089	-2,685	±0,077	-5,317	±0,087
1	-0,166	±0,035	-1,929	±0,092	-3,057	±0,081	-3,219	±0,085	--	--
2	-0,185	±0,049	-2,083	±0,079	-4,135	±0,087	-3,967	±0,082	--	--
5	-0,185	±0,0399	-2,098	±0,062	-4,314	±0,075	-4,323	±0,097	--	--
30	-0,247	±0,0599	-2,136	±0,095	-4,462	±0,088	-4,804	±0,099	--	--
60	-0,333	±0,0797	-2,267	±0,079	-4,732	±0,090	-5,046	±0,096	--	--

Table A3. Data sheet of ClO_2 inactivation of *S. aureus* at varying oxidant concentration

[ClO_2]	**0.50 mg/L**		**1.00 mg/L**		**2.50 mg/L**		**3.50 mg/L**		**5.00 mg/L**	
Contact time(min)	**Log (Nt/No) ± SD**									
0	0		0		0		0		0	
0,5	-0,0732	±0,095	-0,9749	±0,135	-2,0982	±0,359	-3,477	±0,473	-5,8318	±0,189
1	-0,1226	±0,096	-1,0097	±0,157	-2,1689	±0,241	-3,579	±0,783	-6,1369	±0,489
2	-0,1351	±0,078	-1,1153	±0,109	-2,3231	±0,413	-3,8441	±0,393	-6,1749	±0,592
5	-0,1479	±0,059	-1,1503	±0,231	-2,3709	±0,389	-3,9651	±0,277	-6,2023	±0,343
30	-0,1749	±0,043	-1,1641	±0,318	-2,3925	±0,221	-4,0164	±0,453	-6,2469	±0,371
60	-0,1854	±0,087	-1,1858	±0,162	-2,4153	±0,432	-4,174	±0,328	-6,2576	±0,399

Table A4. Data sheet for ClO_2 (2.0 mg/L) inactivation of *E. coli* at varying pH conditions

	pH 6.55		pH 7.00		pH 7.40		pH 8.05		pH 8.55	
Contact time(min)	**Log (Nt/No) ± SD**									
0	0	0	0		0	0	0		0	
0,5	-0,318	±0.018	-2,017	±0,241	-2,483	±0,17	-2,619	±0,31	-3,171	±0,31
1	-0,477	±0.023	-2,049	±0,132	-2,62	±0,28	-2,962	±0,26	-3,787	±0,24
2	-0,487	±0.027	-2,269	±0,199	-2,869	±0,23	-3,182	±0,14	-4,051	±0,21
5	-0,52	±0.031	-2,943	±0,24	-2,979	±0,34	-3,183	±0,25	-4,285	±0,32
30	-0,554	±0.022	-3,071	±0,201	-3,005	±0,15	-3,479	±0,33	-4,528	±0,16
60	-0,619	±0.043	-3,182	±0,15	-3,351	±0,21	-3,737	±0,27	-4,732	±0,29

Table A5. Data sheet for ClO_2 (2.0 mg/L) inactivation of *P. aeruginosa* at varying pH conditions

	pH 6.55		pH 7.00		pH 7.40		pH 8.05		pH 8.50	
Contact time(min)	**Log (Nt/No) ± SD**									
0	0		0		0		0		0	
0,5	-0,072	±0,009	-0,29	±0,034	-0,458	±0,027	-1,203	±0,047	-1,551	±0,017
1	-0,099	±0,007	-0,382	±0,054	-0,471	±0,054	-1,221	±0,036	-1,605	±0,025
2	-0,123	±0,080	-0,481	±0,060	-0,617	±0,064	-1,37	±0,048	-1,647	±0,053
5	-0,222	±0,089	-0,573	±0,073	-0,746	±0,043	-1,384	±0,043	-1,774	±0,042
30	-0,241	±0,076	-0,692	±0,065	-0,882	±0,047	-1,423	±0,060	-1,926	±0,055
60	-0,25	±0,032	-0,75	±0,046	-0,893	±0,039	-1,553	±0,070	-2,078	±0,043

Table A6. Data sheet for ClO_2 (2.0 mg/L) inactivation of *S. aureus* at varying pH conditions water

	pH 6.55		pH 7.00		pH 7.50		pH 8.05		pH 8.50	
Contact time(min)	**Log (Nt/No) ± SD**									
0	0		0		0		0		0	
0,5	-0,373	±0,032	-0,57	±0,045	-0,643	±0,058	-1,809	±0,019	-2,174	±0,045
1	-0,417	±0,047	-0,61	±0,034	-0,925	±0,048	-2,168	±0,045	-2,201	±0,035
2	-0,434	±0,039	-0,75	±0,046	-1,195	±0,039	-2,323	±0,034	-2,263	±0,039
5	-0,573	±0,056	-0,83	±0,063	-1,391	±0,063	-2,394	±0,036	-2,453	±0,043
30	-0,595	±0,039	-0,89	±0,048	-1,472	±0,045	-2,415	±0,046	-2,508	±0,051
60	-0,653	±0,041	-0,96	±0,039	-1,578	±0,052	-2,496	±0,042	-2,575	±0,042

Table A7. Data sheet of ClO_2 (1.0 mg/L) inactivation of *E. coli* at varying temperature conditions

Temp.	4^oC		15^oC		22^oC		30^oC		37^oC	
Contact time(min)	**Log (Nt/No) ± SD**									
0	0		0		0		0		0	
0,5	-2,017	±0,15	-2,138	±0,31	-3,172	±0,27	-4,296	±0,21	-4,422	±0,32
1	-2,049	±0,19	-2,33	±0,24	-3,789	±0,25	-4,508	±0,33	-4,572	±0,38
2	-2,419	±0,22	-2,455	±0,17	-4,051	±0,23	-4,621	±0,29	-4,730	±0,28
5	-2,432	±0,27	-2,574	±0,21	-4,285	±0,24	-5,554	±0,17	-5,708	±0,22
30	-2,573	±0,16	-3,462	±0,29	-4,507	±0,32	-5,804	±0,39	-5,866	±0,34
60	-2,847	±0,24	-3,725	±0,19	-4,732	±0,21	-5,855	±0,25	-5,986	±0,26

Table A8. Data sheet for ClO_2 (2.0 mg/L) inactivation of *P. aeruginosa* at varying temperature conditions

Temp.	4^oC		15^oC		22^oC		30^oC		37^oC	
Contact time(min)	**Log (Nt/No) ± SD**									
0	0		0		0		0		0	
0,5	-0,046	±0,0032	-1,905	±0,16	-2,084	±0,39	-2,318	±0,25	-2,783	±0,23
1	-0,054	±0,0045	-1,928	±0,28	-2,186	±0,21	-2,477	±0,14	-2,802	±0,32
2	-0,084	±0,0037	-2,083	±0,21	-2,198	±0,39	-2,691	±0,16	-2,879	±0,42
5	-0,361	±0,024	-2,098	±0,17	-2,225	±0,18	-2,985	±0,26	-3,037	±0,34
30	-0,394	±0,054	-2,136	±0,35	-2,306	±0,25	-3,156	±0,32	-3,316	±0,43
60	-0,889	±0,061	-2,267	±0,43	-2,341	±0,31	-3,280	±0,41	-3,536	±0,19

Table A9. Data sheet of ClO_2 (2.0 mg/L) inactivation of *S. aureus* at varying temperature conditions

Temp	4^oC		15^oC		22^oC		30^oC		37^oC	
Contact time(min)	Log(Nt/No) ± SD									
0	0		0		0		0		0	
0,5	-1,0117	±0,16	-1,2912	±0,11	-2,0982	±0,22	-3,7109	±0,16	-3,9101	±0,19
1	-1,0877	±,09	-2,0542	±0,21	-2,1689	±0,14	-3,9464	±0,24	-4,19862	±0,23
2	-1,1147	±0,13	-2,0952	±0,11	-2,3231	±0,12	-4,2688	±0,42	-4,4195	±0,44
5	-1,1829	±0,07	-2,1451	±0,26	-2,3709	±0,23	-4,34	±0,31	-4,421	±0,32
30	-1,2296	±0,21	-2,2455	±0,15	-2,3925	±0,21	-4,487	±0,33	-4,5308	±0,27
60	-1,241	±0,15	-2, 3113	±0,23	-2,4143	± 0,432	-4,492	± 0,38	-4,5721	±0,29

Table A10. Data sheet of varying initial densities, N_o, of *E. coli* exposed to ClO_2 (0,75 mg/L)

	N_o=2.61E7	N_o=2.43E6	N_o= 2.32E5
Time(min)		**Log (No/Nt)**	
0	0	0	0
0,5	-2,235	-2,352	-2,221
1	-2,381	-2,487	-2,411
2	-2,476	-2,597	-2,505
5	-2,655	-2,783	-2,861

Table A11. Data sheet of varying initial densities, N_o, of *P. aeruginosa* exposed to ClO_2 (1.0 mg/L)

	No=1.27E5	No=1.36E6	No= 1.74E7
Time(min)		**Log(Nt/No)**	
0	0	0	0
0,5	-2,672	-2,352	-2,122
1	-2,753	-2,415	-2,58
2	-2,82	-2,522	-2,601
5	-2,85	-2,743	-2,654

Table A12. Data sheet of varying initial densities, N_o, of *S. aureus* exposed to ClO_2 (2.5 mg/L)

	No=1.22E5	No=1.04E6	No = 1.13E7
Time(min)		**Log(Nt/No)**	
0	0	0	0
0,5	-2,072	-2,23	-2,23
1	-2,283	-2,41	-2,41
2	-2,341	-2,52	-2,50
5	-2,492	-2,61	-2,59

Table A 13. Data sheet of ONPG hydrolysis reaction with the cells of *E. coli*

	Specific activity in Miller Units (MU)			
1	182,2	55,10	51,00	296,87
5	37,75	10,50	9,96	109,37
10	19,25	5,18	4,75	69,60
15	13,20	3,45	3,20	62,17
20	10.19	2,59	2,15	58,25
25	8,20	2,07	1,98	51,47
30	6,31	1,73	1,54	49,95

Table A14. Data sheet of the effect of ClO_2 treatment on the release of 260 nm absorbing materials from *E. coli*. Absorbance data expressed as a ratio of the ClO_2 treated cells relative to the control (untreated cells) ±SD

[ClO_2]	**0.50 mg/L**		**1.00 mg/L**		**2.50 mg/L**		**5.00 mg/L**	
Time(min)								
0	1		1		1		1	
8	1,076	±0,08	1,26	±0,12	1,422	±0,13	1,539	±0,13
16	1,224	±0,073	1,33	±0,09	1,436	±0,11	1,546	±0,13
24	1,264	±0,11	1,353	±0,11	1,457	±0,14	1,557	±0,22
32	1,289	±0,09	1,36	±0,63	1,472	±0,18	1,567	±0,16
40	1,308	±0,13	1,37	±0,17	1,49	±0,14	1,587	±0,21

Table A15. Data sheet of the effect of ClO_2 treatment on the release of 260 nm absorbing materials from *P. aeruginosa*

$[ClO_2]$/mg/L	**0.50 mg/L**		**1.00 mg/L**		**2.50 mg/L**		**5.00 mg/L**	
Time (min)								
0	1		1		1		1	
8	1,1317	±0,039	1,293	±0,031	1,4586	±0,13	1,7109	±0,034
16	1,1462	±0,034	1,35	±0,092	1,4968	±0,111	1,7963	±0,059
24	1,2025	±0,0511	1,395	±0,071	1,524	±0,050	1,8035	±0,062
32	1,2061	±0,0289	1,416	±0,082	1,5349	±0,045	1,80599	±0,077
40	1,2235	±0,076	1,405	±0,124	1,495	±0,067	1,88073	±0,052

Table A16. Data sheet of the effect of ClO_2 treatment on the release of 260 nm absorbing materials from *S. aureus*

[ClO_2]/mg/L	**0.50 mg/L**		**1.00 mg/L**		**2.50 mg/L**		**5.00 mg/L**	
Time (min)								
0	1		1		1		1	
8	1,076	±0,032	1,260	±0,033	1,422	±0,023	1,539	±0,033
16	1,224	±0,045	1,292	±0,012	1,436	±0,045	1,546	±0,018
24	1,264	±0,056	1,333	±0,019	1,457	±0,035	1,557	±0,054
32	1,289	±0,077	1,353	±0,042	1,485	±0,064	1,567	±0,037
40	1,2908	±0,039	1,351	±0,061	1,505	±0,073	1,593	±0,055

Table A17. Residual concentrations of ozone in water as a function of bubbling time at pH 6.8, temperature (22 ±2^0 C) and flow rate (2.0 L/min) in the presence of 2.5 % organic solvents

	Control		Ethanol		DMSO		Methanol		Ethyl acetate	
Bubbling time(min)	**[ClO_2]/mg/L ± SD**									
2	0,224	±0,013	0,282	±0,015	0,273	±0,018	0,191	±0,045	0,299	±0,025
4	0,378	±0,086	0,415	±0,032	0,429	±0,077	0,293	±0,019	0,462	±0,015
6	0,432	±0,052	0,490	±0,057	0,537	±0,063	0,346	±0,075	0,593	±0,017
8	0,498	±0,027	0,553	±0,078	0,618	±0,017	0,414	±0,029	0,675	±0,014
10	0,565	±0,017	0,688	±0,022	0,629	±0,012	0,468	±0,056	0,747	±0,023
12	0,647	±0,043	0,751	±0,073	0,782	±0,085	0,533	±0,091	0,821	±0,078

Table A18 Residual concentrations of ozone in water as a function of bubbling time at pH 6.8, temperature (22 ±2^0 C) and flow rate (2.0 L/min) in the presence of 5.0 % organic solvents

	Control		Ethanol		DMSO		Methanol		Ethyl acetate	
Bubbling time(min)	**[ClO_2]/mg/L ± SD**									
2	0,224	±0,013	0,301	±0,0142	0,35	±0,0782	0,243	±0,0211	0,388	±0,0254
4	0,378	±0,086	0,469	±0,092	0,52	±0,0467	0,342	±0,0255	0,511	±0,025
6	0,432	±0,052	0,553	±0,032	0,677	±0,0345	0,418	±0,0712	0,652	±0,034
8	0,498	±0,027	0,627	±0,071	0,797	±0,0128	0,483	±0,0115	0,784	±0,022
10	0,565	±0,017	0,798	±0,012	0,853	±0,0871	0,553	±0,0552	0,893	±0,017
12	0,647	±0,043	0,855	±0,088	0,901	±0,0651	0,597	±0,0312	0,985	±0,062

Table A19. Data sheet of bacteria inactivation of ozone bubbled for 12 min at pH 6.8 and (22 ±2) ^{0}C for *E. coli* in 2.5 % organic solvent

Control			Ethanol		DMSO		Methanol		Ethyl acetate	
Contact time(min)	**Log (Nt/No) ± SD**									
0	0		0		0		0		0	
0,5	-1,783	±0,04	-3,455	±0,15	-2,887	±0,34	-2,119	±0,41	-3,977	±0,42
1	-1,951	±0,012	-3,854	±0,082	-3,292	±0,172	-2,231	±0,33	-4,172	±0,36
1,5	-2,059	±0,031	-4,159	±0,31	-3,853	±0,43	-2,275	±0,38	-4,665	±0,35
2	-2,435	±0,022	-4,591	±0,34	-4,493	±0,27	-2,494	±0,06	-5,053	±0,42
3	-2,586	±0,037	-4,636	±0,07	-4,912	±0,42	-2,528	±0,35	-5,612	±0,22
4	-2,671	±0,032	-4,933	±0,41	-5,266	±0,32	-2,614	±0,42	-5,739	±0,15
5	-2,759	±0,08	-5,081	±0,39	-5,357	±0,17	-2,846	±0,45	-5,902	±0,27
6	-2,902	±0,23	-5,232	±0,45	-5,429	±0,09	-2,857	±0,37	-5,947	±0,45

Table A20. Data sheet of bacteria inactivation of ozone bubbled for 12 min at pH 6.8 and (22 ±2) ^{0}C for *E. coli* in 5.0 % organic solvent

Control		Ethanol		DMSO		Methanol		Ethyl acetate	
Contact time(min)	**Log (Nt/No)± SD**								
0	0		0		0		0		0	
0,5	-1,953	±0,11	-3,875	±0,19	-3,173	±0,21	-2,121	±0,22	-4,077	±0,18
1	-2,045	±0,32	-4,554	±0,37	-3,910	±0,09	-2,243	±0,31	-4,734	±0,32
1,5	-2,139	±0,41	-4,912	±0,25	-4,653	±0,07	-2,427	±0,41	-4,992	±0,16
2	-2,336	±0,37	-5,111	±0,18	-4,793	±0,18	-2,592	±0,29	-5,379	±0,40
3	-2,476	±0,22	-5,236	±0,52	-5,012	±0,21	-2,751	±0,32	-5,882	±0,15
4	-2,723	±0,19	-5,543	±0,23	-5,421	±0,32	-2,789	±0,17	-5,973	±0,23
5	-2,799	±0,48	-5,592	±0,34	-5,447	±0,37	-2,823	±0,21	-6,021	±0,28
6	-2,801	±0,301	-5,832	±0,29	-5,621	±0,15	-3,191	±0,18	-6,239	±0,34

Table A21. Data sheet of bacteria inactivation of ozone bubbled for 12 min at pH 6.8 and (22 ±2) ºC for *S. aureus* in 2.5 % organic solvent

	Control		Ethanol		DMSO		Methanol		Ethyl acetate	
Contact time(min)					**Log (Nt/No)**					
0	0		0		0		0		0	
0,5	-0,578	±0,19	-2,318	±0,18	-2,199	±0,37	-0,887	±0,02	-2,174	±0,21
1	-0,987	±0,37	-2,626	±0,32	-2,315	±0,24	-1,183	±0,05	-2,521	±0,09
1,5	-1,379	±0,25	-2,861	±0,16	-2,716	±0,32	-1,429	±0,25	-3,071	±0,07
2	-1,541	±0,18	-2,928	±0,4	-2,805	±0,19	-1,731	±0,14	-3,379	±0,18
3	-1,626	±0,55	-3,165	±0,15	-2,942	±0,35	-1,956	±0,31	-3,844	±0,21
4	-1,713	±0,23	-3,483	±0,23	-3,254	±0,24	-2,007	±0,21	-4,162	±0,32
5	-1,901	±0,34	-3,791	±0,28	-3,476	±0,42	-2,186	±0,28	-4,359	±0,37
6	-1,922	±0,29	-3,842	±0,34	-3,734	±0,33	-2,472	±0,31	-4,435	±0,15

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