STUDIES ON WATERBORNE PATHOGEN REACTIVATION AFTER DISINFECTION

A Dissertation

by

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ABSTRACT

Reactivation of different *Escherichia coli* strains and *Aeromonas hydrophila* after ultraviolet (UV), ultrasound, and photocatalytic disinfection treatments was addressed in this study. Photocatalytic disinfection was carried out under low pressure ultraviolet (LP UV) irradiation at five titanium dioxide (TiO_2) concentrations (1 g/L, 0.5 g/L, 0.75 g/L, and 0.1 g/L) to achieve 5 $\log_{10}$ reduction of a laboratory *E. coli* K-12 strain (ATCC® 10798). Regrowth and reactivation of *E. coli* in dark and light was studied up to 4 h after disinfection period. During the repair period, flow cytometry had shown 4-5 $\log_{10}$ higher cell counts than culture based method. Photocatalysis at 0.1 g/L TiO_2 had resulted in 50% cells with intact cell membrane during the repair period and has lowered the repair rate of the *E. coli* (ATCC® 10798) after disinfection.

Then ultrasound (24 kHz) and UV-C irradiation were applied to inactivate four *E. coli* isolates (ATCC®10798, *E. coli* isolate from feces of feral hog and deer, and treated wastewater effluent) to nearly 8 $\log_{10}$ reduction. Photoreactivation and dark repair of *E. coli* isolates were studied over a 24 h period after disinfection. In general, ultrasound disinfection had resulted in higher inactivation rate of 0.52 log min^{-1} than UV-C (0.39 log min^{-1}) for *E. coli* isolates. The extent of percent log repair of ultrasound inactivated *E. coli* isolates after 24 h of dark repair and photoreactivation were 30% lower than after UV-C. The metabolic activity of *E. coli* cells was greatly reduced after ultrasound as shown by AlamarBlue® assay. Transmission electron micrographs of ultrasound disinfected *E. coli* revealed shearing and size reduction of bacterial cells.

Aeromonas hydrophila (ATCC® 35654), an emerging pathogen, was inactivated using a 24 kHz continuous ultrasound and UV-C in combination with three TiO_2 concentrations (1g/L, 0.1 g/L and 0 g/L). High inactivation rate of 1.52 log min^{-1} was observed for ultrasound disinfection in absence of TiO_2. *Aeromonas hydrophila* had showed a net log reduction of 6 log_{10} after ultrasound exposure in comparison to a net 2 log_{10} reduction after UV-C over a 24 h repair period. Metabolic activity of *Aeromonas hydrophila* was adversely affected by ultrasonication as well. Ultrasound appears to be effective in inactivating environmental *E. coli* isolates and *Aeromonas hydrophila* in water through shearing and breaking effects, which decreased the metabolic activity as well as photoreactivation and dark repair.

DEDICATION

I would like to dedicate this work as a thanksgiving to the Lord for all his blessings that he has showered upon me, without which this work would not have been possible. I would also like to dedicate this work to my mother, Ranjeet Kaur; my mother-in-law, Jasbir Kaur; my father, Charanjeet Singh; my brother, Harsimran Singh; my late Grandmother; my late Grandfather and my loving husband, Sukhdeep Singh, for all of their love, support, and prayers.

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CHAPTER I

INTRODUCTION

1.1 Background

Water plays an essential role in sustaining all forms of life on Earth, with only 3% of the total water reserves as fresh water (Oppenländer 2003). Quality of drinking water has always been an issue, as there have been many reported deaths due to presence of pathogenic organisms, organic compounds, heavy metals, and other contaminants. Bacteria are the leading cause of impairment of surface waters, including rivers, lakes, and streams in the U.S. (USEPA 2008). This problem is more severe in the developing countries which lack infrastructure for water treatment and have high rate of mortality mainly among young children from preventable water related disease (Souter *et al*. 2003). Provision for providing safe drinking water has always been an utmost issue. More than one billion people do not have access to safe and adequate drinking water sources as estimated by the World Health Organization (Souter *et al*. 2003).

Inactivation of pathogenic microorganisms is the goal of water disinfection processes. Many disinfection processes have evolved during past few decades such as ozonation, solar disinfection, photocatalysis, and ultraviolet (UV) disinfection. UV disinfection has gained popularity due to high inactivation rate with low disinfectant doses and no disinfection by-products as compared to chlorine disinfection (Boorman *et al*. 1999; Hua and Thompson 2000; Liu and Yang 2003; Galvez *et al*. 2007; Quek and Hu 2008; Drakopoulou *et al*. 2009). Residual amount of chlorine in water after

chlorination prevents the re-growth of microorganisms which enables its wide application. The major drawback of chlorination is the disinfection by-products which are carcinogenic in nature (Galvez *et al*. 2007). Ozone is highly reactive but very unstable in water. Moreover, ozonation is an expensive process (Gunten 2003; Ibanez 2007; Malato *et al*. 2007).

Disinfection using UV radiation inactivates the microorganisms primarily through the effects of UV-C radiation of 220-280 nm wavelengths. High energy wavelengths denature the bacterial DNA; thereby hindering the DNA replication which results in the inactivation of the microorganisms. Ultraviolet disinfection being a physical process is not affected by pH and temperature (Quek and Hu 2008). Upon absorption of high energy wavelength emitted from UV source, pyrimidine dimers, namely *cis-syn* cyclobutane are formed in bacterial cells (Hallmich and Gehr 2010). It has been shown that microorganisms have evolved with photoreactivation and dark repair mechanisms, when subjected to near UV or visible light wavelength and dark conditions, respectively (Quek and Hu 2008; Drakopoulou *et al*. 2009; Shang *et al*. 2009; Hallmich and Gehr 2010). These repair pathways reverse the UV damage on the DNA by repairing the pyrimidine dimers (Figure 1.1) (Lindenauer and Darby 1994; Quek and Hu 2008). Reactivation of microbial cells is a major problem of concern for the water treatment plants employing UV disinfection. This could potentially impact the overall efficiency of the treatment and the safe delivery of drinking water.

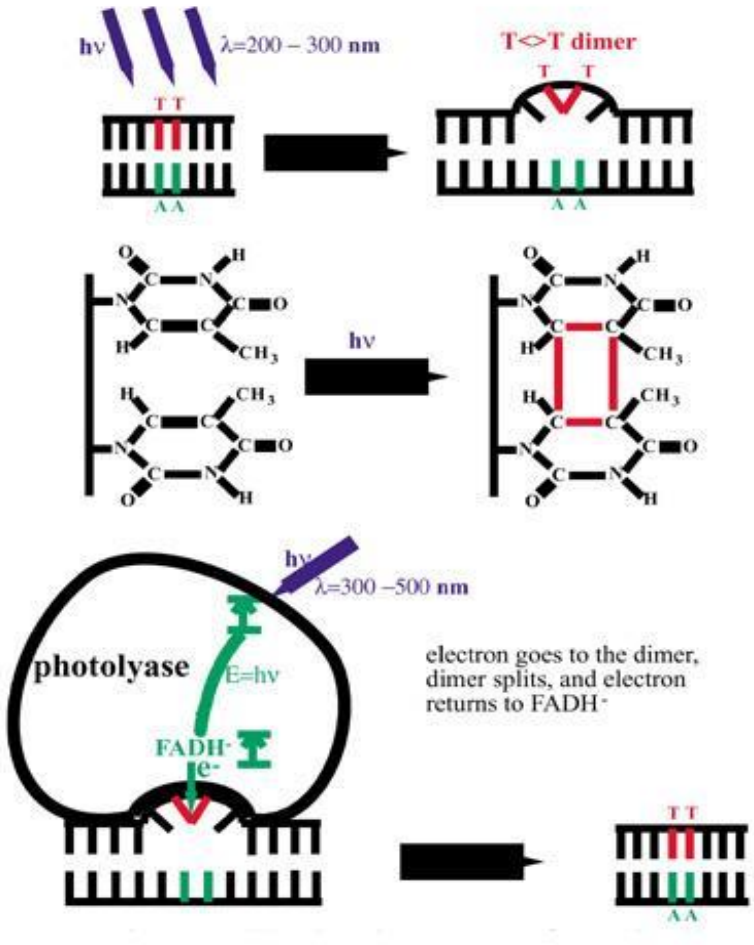
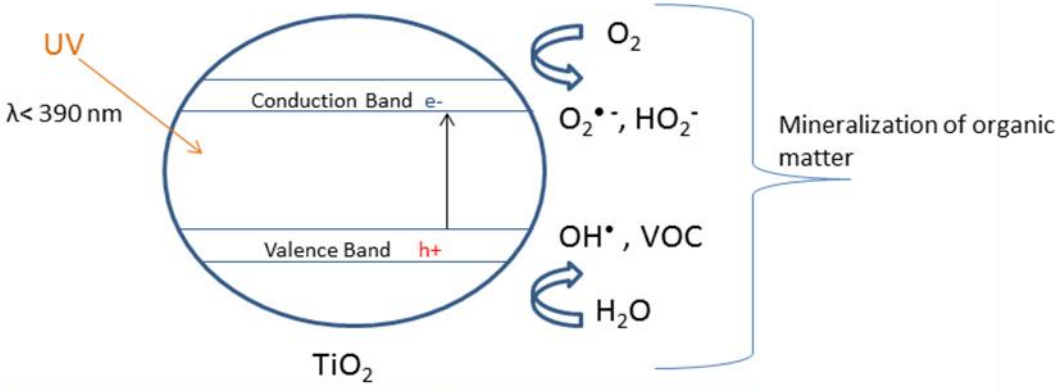


Figure 1.1 Photo-repair of pyrimidine dimer formed in DNA molecule during ultraviolet irradiation (Source: http://stuchebrukhov.ucdavis.edu).

In recent years, photocatalytic oxidation processes have emerged out as cheap and effective means of water and wastewater disinfection (Liu and Yang 2003). Titanium dioxide (TiO_2) is the most widely studied photocatalyst in water disinfection (Ibanez 2007; Theron *et al*. 2008). Advanced photocatalytic processes involve generation of highly reactive species in water such as hydroxyl radicals, hydrogen peroxide, valence band holes, and conduction band electrons (Figure 1.2), which enable

degradation of organic compounds and cell lysis (Galvez *et al*. 2007). Photocatalytic reaction might cause fatal cell damage and disturbance of vital functions and structures, thereby facilitating the inactivation of microorganisms.



Process	Name
$TiO_2 + h\upsilon \rightarrow h_{VB}^+ + e_{CB}^-$	Charge Separation
$h_{VB}^+ + H_2O \rightarrow OH^\bullet$	$OH^\bullet$Generation (VB)
$e_{CB}^- + O_2 \rightarrow O_2^{\bullet-} + H_2O \rightarrow OH^\bullet$	$OH^\bullet$Generation (CB)

Figure 1.2 Titanium dioxide induced photocatalysis mechanism.

Photocatalytic processes using TiO_2 have been studied on *Escherichia coli* (*E. coli*), a Gram-negative bacterium. *E. coli* is a good microbial indicator of fecal pollution. It is mostly employed as an indicator organism due to its well studied growth conditions, easy propagation and detection in the laboratory conditions (Ibanez 2007, Maier *et al*. 2009). Most of the disinfection studies were focused on laboratory *E. coli* strain bacteria (Lindenauer and Darby 1994; Sanz *et al*. 2007; Quek and Hu 2008; Shang *et al*. 2009; Hallmich and Gehr 2010). There is a need to analyze the treatment efficiency on

environmental *E. coli* strains. Reactivation mechanisms and kinetics of bacteria after disinfection has not been studied much.

Disinfection of emerging pathogens should be studied as well. One such emerging pathogen in drinking water is *Aeromonas hydrophila.* It is categorized as an emerging pathogen from the contaminant candidate list provided by the US Environmental Protection Agency (Chauret *et al*. 2001; Silvestry-Rodriguez *et al*. 2007; Chopra *et al*. 2009). *Aeromonas hydrophila*, a Gram negative bacterium is a primary fish pathogen and an opportunistic human pathogen (Fernández *et al*. 2000; Chauret *et al*. 2001; Chopra *et al*. 2009; Khan *et al*. 2012). Even at low concentrations of 1 to 10^4 CFU/100 mL in drinking water, it had caused several gastroenteritis outbreaks (Fernández *et al*. 2000; Silvestry-Rodriguez *et al*. 2007; Lontsi *et al*. 2013). *Aeromonas* spp. found in chlorinated domestic water supply were considered to be potentially enteropathogenic (Sisti *et al*. 1998; Massa *et al*. 1999; Chauret *et al*. 2001; Chopra *et al*. 2009).

Possible future outbreaks of waterborne diseases due to attachment of *Aeromonas hydrophila* with biofilms in drinking water supplies is a major concern (Fernández *et al*. 2000; Furuta *et al*. 2004; Lontsi *et al*. 2013). Wide use of chlorination has resulted in surfacing of resistant microorganisms, such as *Aeromonas* and *Cryptosporidium* (Furuta *et al*. 2004). Other disinfection treatments such as ozonation, ultraviolet, and ionizing radiations are being used instead of chlorination, but they are ineffective in decontaminating emerging chlorine resistant microorganisms such as

Aeromonas spp. (Furuta *et al*. 2004). Thus development of effective and alternative disinfection methods for inactivation of *Aeromonas hydrophila* is much needed.

Ultrasound disinfection is an attractive means to enhance water quality as it is simple and causes no toxic by-products (Furuta *et al*. 2004). Use of ultrasound for wastewater disinfection, in combination with UV, hydrogen peroxide and/or ozone have been studied and demonstrated in few recent studies (Hua and Thompson 2000; Paleologou *et al*. 2007; Drakopoulou *et al*. 2009). Mechanical vibrations of frequencies between 20 kHz and 800 kHz result in propagation of ultrasound waves. When these waves propagate into liquid media, alternating compressions and rarefactions are produced. When the amplitude of the ultrasound wave is high enough, cavitation occurs (Cameron *et al*. 2008). Acoustic cavitation caused by ultrasonication helps in mechanically disrupting the biological cells. High power ultrasound at low frequencies is an effective means for disintegrating bacterial cells (Piyasena *et al*. 2003; Cameron *et al*. 2008). Immediate implosion of cavities results in formation of high pressure regions, which disrupts the biological cells. It also enables the breakdown of clusters of microorganisms formed which protect the inner microorganisms from degradation (Joyce *et al*. 2003; Blume and Neis 2004; Furuta *et al*. 2004; Paleologou *et al*. 2007; Cameron *et al*. 2008; Drakopoulou *et al*. 2009).

In addition, ultrasonication facilitates interaction of short lived hydroxyl radicals, continuing further oxidation of the organic compounds and biological cells (Piyasena *et al*. 2003; Drakopoulou *et al*. 2009). Ultrasonic waves have also been considered as an alternative energy source for excitation of TiO_2 band gap (Kubo *et al*. 2005;

Drakopoulou *et al*. 2009). Kubo *et al*. (2005) evaluated the ultrasound disinfection of *E. coli* in presence of TiO_2. Hua and Thompson (2000) investigated practicality of ultrasound irradiation as a physical-chemical method for disinfection of *E. coli* in water. They investigated the effect of ultrasound frequency, dissolved gas, and acoustic power intensity on the inactivation of *E. coli* during sonolysis (Hua and Thompson 2000). Though reference strains of bacteria are studied extensively, but not much work has been done on evaluating the disinfection treatments on environmental strains. Moreover, application of ultrasound and ultraviolet (UV-C) along with photocatalysis on inactivation of waterborne pathogens have not been thoroughly studied.

Most of the disinfection studies have used culture based method (plating method) for bacterial enumeration. The plating technique allows distinction between culturable and unculturable cells. However, some of the uncultured cells could be viable but non culturable (VBNC) and thus, are not detected by conventional plating method. Moreover, the plating method such as heterotrophic plate counts underestimates the bacterial counts at least by two orders of magnitude (Egli 2008). On the other hand, limitations of culture based method were overcome by molecular techniques. One such technique is flow cytometry, which provides a clear distinction between intact cell membrane (live) and compromised cell membrane (dead) cells with use of specific dye stains, like SYTO9 and propidium iodide (PI) (Berney *et al*. 2007; Hammes *et al*. 2008).

Flow cytometry along with use of fluorescent dye is a leading edge technology extensively used in the bacterial enumeration studies. Cells stained with SYTO9 and PI results in three regions: live, dead, and intermediate (Berney *et al*. 2007). Cells with

compromised cell membranes are considered dead and thus, there is a low probability of replication as they are not viable. The intermediate states could be attributed by the extent of damage caused by the disinfectant on the microorganism. The microorganisms at the intermediary state could potentially revive once exposed to vital growth conditions. Flow cytometry can distinguish between viable and non-viable cells, in contrast to conventional plating method. This distinction is very crucial for determining the overall disinfection system efficiency in killing pathogens as some could be viable but uncultured.

The other method of assessing the cell health is through determining the cell viability and metabolic activity. Accurate method of accessing the cell damage by determining the cell viability and metabolic activity should be incorporated as conventional culture based has limitations in determining the cell viability. There are many non-toxic vital dyes and tests available for cell viability determination. One of such vital dye which is non-toxic to cells is AlamarBlue®. The AlamarBlue® cell viability assay will enable in determining the metabolic state of the cells. It has a higher sensitivity and reproducibility in detection of low cell concentrations (O'Brien *et al*. 2000; Al-Nasiry *et al*. 2007). The active reagent of AlamarBlue®, resazurin will be reduced to resorufin by healthy cells. Till now, there has not been enough work done on assessing the cell viability using AlamarBlue® dye to study the effectiveness of disinfection treatment.

1.2 Objectives

The overall goal of this research work was to study the reactivation of *E. coli* strains and *Aeromonas hydrophila* in water after being exposed to UV, ultrasound, and photocatalytic disinfection. The extent of photoreactivation and dark repair reactivation mechanisms were evaluated for these microorganisms. The bacteria were enumerated by a culture based method and flow cytometry, and were assayed for metabolic activity using AlamarBlue® assay to assess the disinfection. The specific objectives were as follows:

Objective 1: Effect of titanium dioxide on reactivation mechanisms of *E. coli* after UV irradiation and photocatalysis. Batch reactors were used to test the following hypotheses:

A) Addition of TiO_2 will reduce the repair rate after disinfection

B) Higher TiO_2 concentration will reduce the repair rate

Objective 2: Assessment of reactivation mechanisms of laboratory *E. coli* strain (ATCC® 10798) and environmental *E. coli* isolates after UV and 24 kHz continuous ultrasound. Batch reactors were used to test the following hypotheses:

A) Ultrasound will reduce the reactivation of *E. coli* isolates in comparison to UV-C disinfection by causing shearing of *E. coli* cells

B) Ultrasound will lower the metabolic activity of the *E. coli* cells in comparison to UV disinfection

Objective 3: Effects of 24 kHz continuous ultrasound, UV irradiation, and photocatalysis on reactivation mechanisms of *Aeromonas hydrophila* (ATCC® 35654) after disinfection. Batch reactors were used to test the following hypotheses:

A) Ultrasound will significantly decrease *Aeromonas hydrophila* reactivation in comparison to UV and UV induced photocatalysis

B) Presence of TiO_2 during ultrasound and UV will enhance the inactivation rate and lower the repair rate

1.3 Rationale and Significance

The repair pathways reverse the UV damage on the DNA by repairing the pyrimidine dimers (Lindenauer and Darby 1994; Quek and Hu 2008). This could potentially impact the overall efficiency of the UV disinfection system. The extent of bacterial reactivation after disinfection should be considered while selecting an appropriate retention time in the water distribution systems. The time spent in the pipes before the final use of disinfected water should take into consideration the repair of bacteria under dark conditions as well. The repair rates and doubling time of waterborne pathogens should be considered in designing the optimum exposure time during disinfection and the lead time involved before the final use of the treated water, whether drinking or treated wastewater. A clear distinction between the viable, non-viable, cultured and uncultured cells is very critical while determining the water quality of highly sensitive distribution areas such as hospitals, nursing homes, healthcare facilities, and pharmaceutical and food manufacturing industries. Culture based methods underestimate the concentration of pathogens while molecular methods will provide more realistic numbers (Hammes and Egli 2010). To avoid potential health risk, further improvement in the water disinfection systems will be required to maintain an effective level of pathogen reduction.

CHAPTER II

ASSESSMENT OF *ESCHERICHIA COLI* REACTIVATION AFTER PHOTOCATALYTIC WATER DISINFECTION USING FLOW CYTOMETRY: A COMPARISON TO CULTURE BASED METHOD[*]

2.1 Overview

Photocatalytic process generates highly reactive oxidative species, such as hydroxyl radicals, which enables oxidation of cellular compounds. Microorganisms often tend to lose their culturability after disinfection, but could remain viable to proliferate under optimum conditions. Estimation of bacterial counts using culture based methods pose limitations in differentiating viable, non-viable, and viable but non-culturable (VBNC) cells. Presence of viable, VBNC state cells in disinfected water could pose a potential health risk and accurate estimation of these cells through molecular method is critical. Assessment of live/dead states of an indicator waterborne pathogen, *Escherichia coli* (ATCC® 10798) after disinfection was conducted using flow cytometry. Photocatalysis was carried out under low pressure ultraviolet (LP UV) at five titanium dioxide (TiO_2) concentrations (1 g/L, 0.5 g/L, 0.75 g/L, and 0.1 g/L). During the repair period, flow cytometry showed 4-5 $\log_{10}$ higher cell counts than culture based method. Photocatalysis using 0.1 g/L TiO_2 had resulted in 50% cells with intact cell

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membrane during the repair period and has lowered the repair rate of the *E. coli* (ATCC® 10798) after disinfection.

2.2 Introduction

Water disinfection using ultraviolet (UV) rays is becoming a popular treatment option because of high inactivation rates with low doses and no carcinogenic by-products formation as compared to other disinfection methods (Lindenauer and Darby 1994; Boorman *et al*. 1999; Liu and Yang 2003; Galvez *et al*. 2007; Quek and Hu 2008; Shang *et al*. 2009; Hallmich and Gehr 2010). Most commonly used UV lamps are low pressure (LP) and medium pressure (MP) mercury lamps. LP UV lamps emit monochromatic UV radiation at 254 nm which is close to the germicidal wavelength of 260 nm, whereas, MP UV lamps emit a broad spectrum wavelengths ranging from 200-400 nm (Quek and Hu 2008). Upon absorption of high energy radiation, microbial DNA is altered via formation of pyrimidine dimers, namely: cis-syn cyclobutane pyrimidine dimers and other photo-products which inhibit DNA replication (Lindenauer and Darby 1994; Quek and Hu 2008; Shang *et al*. 2009; Hallmich and Gehr 2010).

However, microorganisms have evolved with repair mechanisms such as photoreactivation and dark repair, which are able to reverse the UV induced damage to DNA (Lindenauer and Darby 1994; Sanz *et al*. 2007; Quek and Hu 2008; Shang *et al*. 2009; Hallmich and Gehr 2010). Photoreactivation involves photoreactivating light in the wavelength of 330-480 nm to activate photolyase enzyme, which enables in splitting of the CPD back to its original monomerized state (Sanz *et al*. 2007; Shang *et al*. 2009). In general, dark repair involves nucleotide excision of damaged DNA which requires

synchronization of dozens of proteins to repair and excise pyrimidine dimmers. Light independent repair of the damaged DNA is termed as dark repair mechanism. The reactivation of microorganisms by either means affect the overall efficiency of the UV disinfection system (Sanz *et al*. 2007; Quek and Hu 2008; Shang *et al*. 2009).

In recent years, photocatalytic oxidation processes have emerged out as cheap and effective means of water and wastewater disinfection (Liu and Yang 2003; Ibanez 2007; Theron *et al*. 2008; Shang *et al*. 2009; Chong *et al*. 2010). Advanced photocatalytic processes generate highly reactive species such as hydroxyl radicals in water, which enables mineralization of cellular organic compounds and inactivation of microorganisms (Dunlop *et al*. 2002; Liu and Yang 2003; Galvez *et al*. 2007; Ibanez 2007; Malato *et al*. 2007; Chong *et al*. 2010). Among the semiconductor catalysts, titanium dioxide (TiO_2) has gained lot of interest, as it is the most active photocatalyst in the energy range of 300 nm - 390 nm (Dunlop *et al*. 2002; Galvez *et al*. 2007; Reddy *et al*. 2008; Chong *et al*. 2010). The energy range for excitation of TiO_2 falls in the UV radiation (200-400 nm), thus a combination of UV and TiO_2 may result in oxidation of cellular compounds and proteins through photocatalysis as well as DNA damage due to UV (Liu and Yang 2003; Shang *et al*. 2009; Chong *et al*. 2010). The reactivation kinetics of bacteria after photocatalysis should be studied to effectively determine the subsequent reactivation after disinfection.

Conventional culture based method poses limitations in effective determination of membrane oxidation, cell state and cell viability. The culture based method does not identify active, inactive, and dead bacteria (Berney *et al*. 2008). Also, it usually

underestimates bacterial counts by at least 2 $\log_{10}$ orders of magnitude and is unable to detect viable but non culturable (VBNC) state cells (Egli 2008; Hammes *et al*. 2008; Hammes and Egli 2010). The combination of flow cytometry with fluorescent stains has been applied to characterize bacterial cells based on membrane integrity, cell state, and conditions. A clear distinction between viable and non-viable cells, in contrast to conventional plating method will be possible with flow cytometry (Berney *et al*. 2007; Berney *et al*. 2008; Hammes *et al*. 2008). Estimation of VBNC state cells in disinfected water would be helpful for assessing the disinfection efficacy, as some microorganisms lose their cultivability after stress conditions.

Thus, in this study, the effect of TiO_2 based photocatalysis on reactivation potential of a non-pathogenic *E. coli* strain (*Escherichia coli* K-12 (ATCC® 10798)) was evaluated. In general, *E. coli* is widely used as an indicator of fecal contamination. It was hypothesized that photocatalytic disinfection would decrease the subsequent photoreactivation and dark repair after disinfection. Also, higher titanium dioxide concentration would decrease the rate of *E. coli* (ATCC® 10798) cell repair. Assessment of flow cytometry along with nucleic acid stains to effectively enumerate live/dead states of *E. coli* during reactivation period was studied. Membrane viability assay was utilized to assess the cell membrane permeability caused during disinfection treatment. A comparison to culture based method was also analyzed to assess the repair potential of bacteria after disinfection. Results from this laboratory studies were presented in this manuscript.

2.3 Materials and Methods

2.3.1 Titanium dioxide stock solution

Stock solution of titanium dioxide (10 g/L) was prepared by mixing 2 g of titanium dioxide powder (Degussa P25, Fisher Scientific, USA) in 200 mL nuclease free deionized (DI) water. The stock solution was stored in dark at room temperature. Before each experiment, the unsterilized stock solution was sonicated for 3 min to ensure homogenous suspension of titanium dioxide.

2.3.2 Bacterial strains and growth

E. coli K-12 (ATCC® 10798) strain was inoculated in 50 mL of Luria broth (Sigma Aldrich, USA) by transferring one loop from the culture stock and incubated at 37°C for 22-24 h until the cell count reached 10^8 CFU/mL. The growth media was continuously mixed to ensure proper distribution of nutrients to the organisms. From the growth media, 20 mL culture was added to a 200 mL working volume in 250 mL glass beaker to achieve 10^{-1} dilution of the culture. All glassware, nuclease free deionized (DI) water and media used for bacterial growth were autoclaved at 121°C for 20 min prior to each experiment.

2.3.3 Photoreactor setup

A UV-C low pressure (LP) germicidal lamp (Bryant energy, USA) with a wavelength of 254 nm was mounted horizontally in a polyvinyl chloride (PVC) box of dimension 81 × 61 × 51cm (L × B × H). Three 250 mL Pyrex® beakers (7.62 cm in height and 6.35 cm in diameter) containing 200 mL water sample and magnetic stir bars, were placed on a magnetic stirrer under LP UV lamp. The average UV irradiance

reaching the irradiated sample was calculated using the Morowitz equation (Morowitz 1950; Hallmich and Gehr 2010):

$$I_{avg} = I_o \left(\frac{1 - e^{-d \times \alpha}}{d \times \alpha} \right) \quad (2.1)$$

Where, I_o is incident irradiance (mW/cm^2); d is water depth under UV irradiation (d= 6.35 cm); α = ln (1/T), T is transmittance at 254 nm with cell path length of 1 cm; I_{avg} is the average irradiance reaching the sample (mW/cm^2). The average light intensity (irradiance) measured before sample exposure using a radiometer ranged from 220 to 245 μW/cm^2. The required UV dose was calculated using the following equation by adjusting the exposure time to achieve 5 log reductions for all the treatments (Hallmich and Gehr 2010).

UV dose (mW. s/cm^2) = I_{avg} (mW/cm^2) × time (s) (2.2)

2.3.4 Batch disinfection study

All the experiments were conducted in triplicates at average pH 6.5 and 22±2°C to ensure their reproducibility. Photoreactivation and dark repair studies were conducted on different days. For each experiment, 200 mL of working volume was used with 0.34% (w/v) salinity. To the above mixture, a desired volume from TiO_2 stock solution and 20 mL of the bacterial culture was added. The bacterium was not washed before addition in order to mimic typical water and treated wastewater conditions, and to avoid any stress on bacteria due to centrifugation. The reactors were stirred at 400 rpm to ensure complete mixing. Photocatalytic disinfection was carried out at 1 g/L, 0.5 g/L, 0.75 g/L, and 0.1 g/L TiO_2 concentrations in suspension subjected to LP UV irradiation.

Disinfection without TiO_2 exposed to UV-C irradiation was also studied. Two dark controls: one with 1 g/L TiO_2 and other without TiO_2 were prepared to study the presence of inactivated or unexcited TiO_2 on bacteria for any lethal damages. After the required 5 log_{10} reduction in cell counts, water samples were immediately exposed either to visible light with average intensity of 30,000 lux or covered with aluminum foil (dark conditions) for 4 h, respectively. The photoreactivation and dark repair was studied to determine the effect of TiO_2 in suppressing the repair after LP UV-TiO_2 disinfection. Regular time interval samples were taken and bacterial enumeration was done by spread plate method and flow cytometry.

2.3.5 Bacteria enumeration

Culture based method

E. coli concentrations were determined by the spread plate method. For this method, appropriate dilutions were performed in de-ionized and nuclease free water and 100 µL of the diluted sample was spread plated on the MacConkey agar plates. The plates were incubated for 24 h at 37±1°C. After incubation, *E. coli* colonies were counted and averaged for the triplicate plates and recorded as colony forming units (CFU/mL).

Flow-cytometric measurements

E. coli cells with intact (live) and compromised cell membrane (dead) were also determined using a FACS Calibur (Becton Dickinson Immuno cytometry Systems, San Jose, CA) flow cytometer, equipped with a 15 mW air-cooled argon laser, using CellQuest (Becton Dickinson) acquisition software. LIVE/DEAD Baclight® bacterial

viability and counting kit (Invitrogen, USA) was used for differentiating the live and dead cells. For the flow cytometric measurement, 10 µL of the water sample from the reactors was taken in 12 × 75 mm (H × D) BD Falcon tube (Fisher Scientific, USA). To this, components from the Baclight® kit were added. A final concentration of 30 µM and 5 µM for PI and STYO9, respectively, was used for the analysis. Green fluorescence from SYTO9 was collected through a 530/30-nm bandpass filter and red fluorescence from PI through a 670 nm longpass filter.

The instrument threshold was set on SYTO9 fluorescence. List mode data were acquired on a minimum of 10,000 viable cells defined by light scatter gates and SYTO9 staining. Data analysis was performed in FlowJo (version 8.8.7, Treestar, Inc., Ashland, OR). Bacterial cells were defined by sequential gates, first on their light scattering properties (forward versus side scatter) then on their SYTO9 staining patterns (SYTO9 versus side scatter).

2.3.6 Repair assessment

In order to assess the recovery of *E. coli* during photoreactivation and dark repair, two parameters, namely: percent log repair and repair rate were examined. To negate the variability in initial cell counts and log reduction after treatment, the following formula was used (Quek and Hu 2008):

$$\% \text{ log repair} = \frac{(N_{initial} - N_0)}{(N_t - N_0)} \times 100 \qquad (2.3)$$

Where N_t is log CFU/mL of *E. coli* at time of exposure, t, to photoreactivation/dark repair conditions; N_o is log CFU/mL of *E. coli* immediately after

LP UV disinfection; and $N_{initial}$ is the initial concentration of *E. coli* before LP UV irradiation (log CFU/mL). The rate of repair was calculated for the first hour of repair using the following equation (Quek and Hu 2008):

$$\text{Rate of repair } \left(\log h^{-1}\right) = \frac{(N_t - N_0)}{t} \quad (2.4)$$

Where N_1 is log CFU/mL of *E. coli* after 1 h of phtotoreactivation/dark repair and t is time interval between two samples, here 1 h.

2.3.7 Statistical analysis

Response in terms of CFU/mL for culture based method and counts/mL for flow cytometry was recorded for each treatment and the data was analyzed using Design Expert®. The full factorial model was used to analyze the data from culture based and flow cytometry methods. A two factor model with different levels each was selected in Design Expert®. Factor A was titanium dioxide concentration with five levels and factor B was time with five levels (photoreactivation and dark repair period). A total number of nine data points were chosen for the statistical analysis. Type I error value of 0.05 was selected for conducting ANOVA.

2.4 Results and Discussion

2.4.1 Culturable *E. coli* cells after LP UV and LP UV-TiO_2 disinfection: culture based method

The average initial concentration of *E. coli* ($N_{initial}$) was 10^7 CFU/mL and after LP UV-TiO_2 disinfection, the concentration was reduced to an average value of 10^2 CFU/mL. The LP UV-TiO_2 treatments had caused nearly 5 $\log_{10}$ reductions for all the

treatments. During the repair period, *E. coli* concentrations were increased to an average value of 10^4 CFU/mL (Figure 2.1). The photoreactivation and dark repair for all the treatments had caused nearly 2 $\log_{10}$ increase in the *E. coli* counts. This resulted in a 3 $\log_{10}$ net reduction after accounting repair period. So, in order to achieve 5 $\log_{10}$ reduction, a disinfection treatment should be designed to achieve 7 $\log_{10}$ reduction during the exposure time.

These results did not support the hypothesis that addition of TiO_2 would lower the repair after disinfection. Presence of TiO_2 did not drastically reduce the *E. coli* repair in comparison to LP UV (Figure 2.1). Higher TiO_2 concentration would reduce the subsequent repair was also not supported. Data in Table 2.1 show that 0.1 g/L TiO_2 had the lowest repair rate of 0.87 log h^{-1} and 0.95 log h^{-1} during photoreactivation and dark repair period, respectively. The percent log repair in presence of 0.1 g/L TiO_2 was the lowest among all other treatments during 4 h of repair (Figure 2.2). Photocatalysis at 0.1 g/L TiO_2 under LP UV had resulted 17.2% dark repair of cells with damaged DNA, whereas, the remaining *E. coli* cells had their cellular compounds and membrane oxidized beyond repair. The percent repair during photoreactivation was slightly lower than dark repair period for 0.1 g/L TiO_2 (Table 2.1).

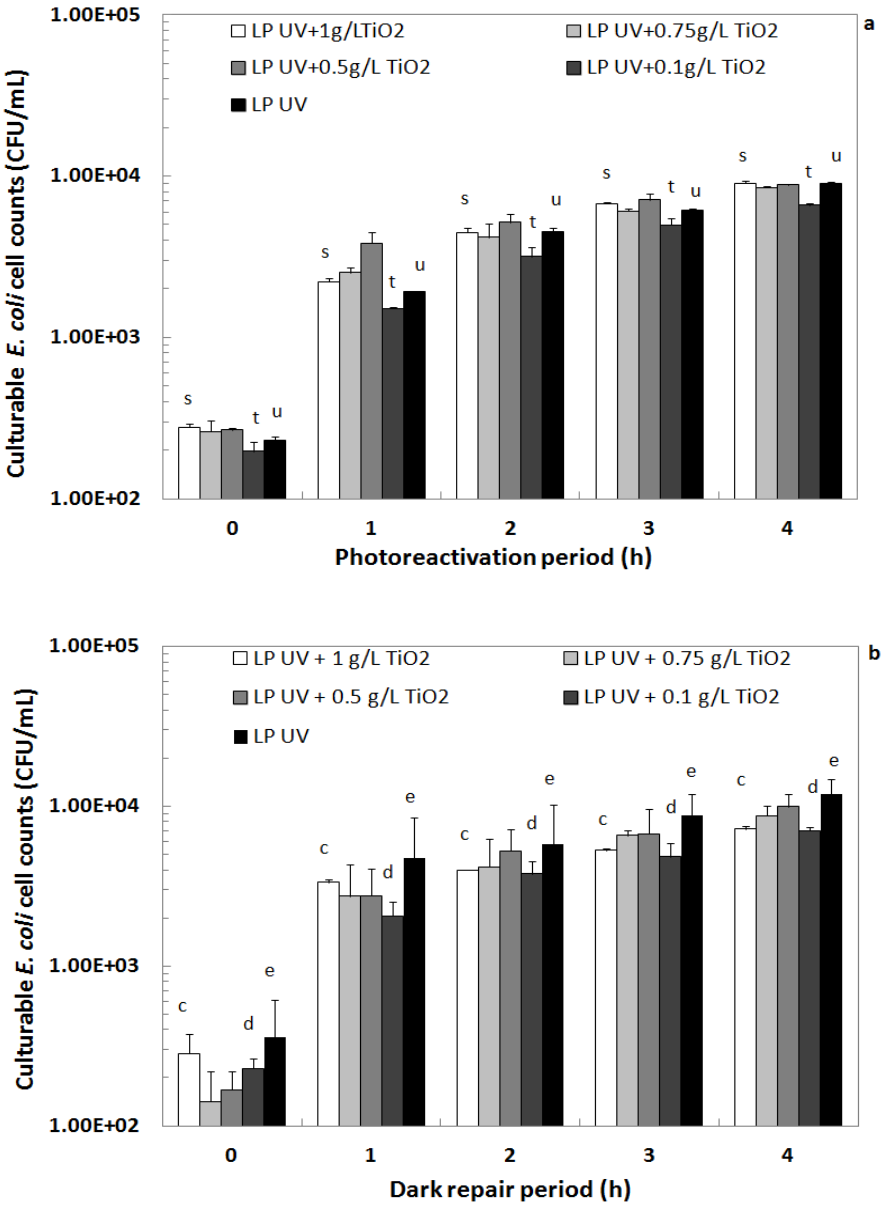


Figure 2.1 Culturable *E. coli* cell counts during 4 h of repair period after LP UV and LP UV-TiO_2 disinfection as determined by culture based method: (a) shows photoreactivation period (*s* and *u*, and *t* and *u* are significantly different, $p = 0.0072$ and $p < 0.0001$, respectively) and (b) shows repair during dark period (*c* and *d*, and *d* and *e* are significantly different, $p = 0.0071$). Standard deviations of three experiments are presented as error bars.

Table 2.1 Culture based assessment of *E. coli* (ATCC® 10798) log reduction, percent log repair, and repair rate for different disinfection treatments for (a) photoreactivation and (b) dark repair

Treatments	Log reduction [1]		% log repair [†]		Repair rate (log h^{-1}) [†]	
	a	b	a	b	a	b
LPUV + 1g/L TiO_2	5.37 ± 0.15[§]	5.27 ± 0.29[§]	16.80 ± 0.39[§]	20.28 ± 1.46[§]	0.90 ± 0.01[§]	1.07 ± 0.12[§]
LPUV + 0.75 g/L TiO_2	5.32 ± 0.07[§]	5.50 ± 0.15[§]	18.50 ± 1.04[§]	23.42 ± 2.45[§]	0.98 ± 0.04[§]	1.29 ± 0.17[§]
LPUV + 0.5 g/L TiO_2	5.36 ± 0.02[§]	5.28 ± 0.10[§]	21.60 ± 1.66[§]	22.97 ± 5.85[§]	1.16 ±0.09[§]	1.21 ± 0.28[§]
LP UV + 0.1 g/L TiO_2	5.38 ± 0.13[§]	5.56 ± 0.01[§]	16.20 ± 0.52[§]	17.16 ± 0.16[§]	0.87 ± 0.03[§]	0.95 ± 0.01[§]
LPUV	5.29 ± 0.12[§]	5.09 ± 0.38[§]	17.50 ± 0.81[§]	22.03 ± 9.75[§]	0.92 ± 0.02[§]	1.12 ± 0.56[§]

[1]viable cell count log reduction during disinfection.
[†]Four hour repair period.
[a]photoreactivation.
[b]dark repair.
[§]Standard deviations of three experiments are presented.

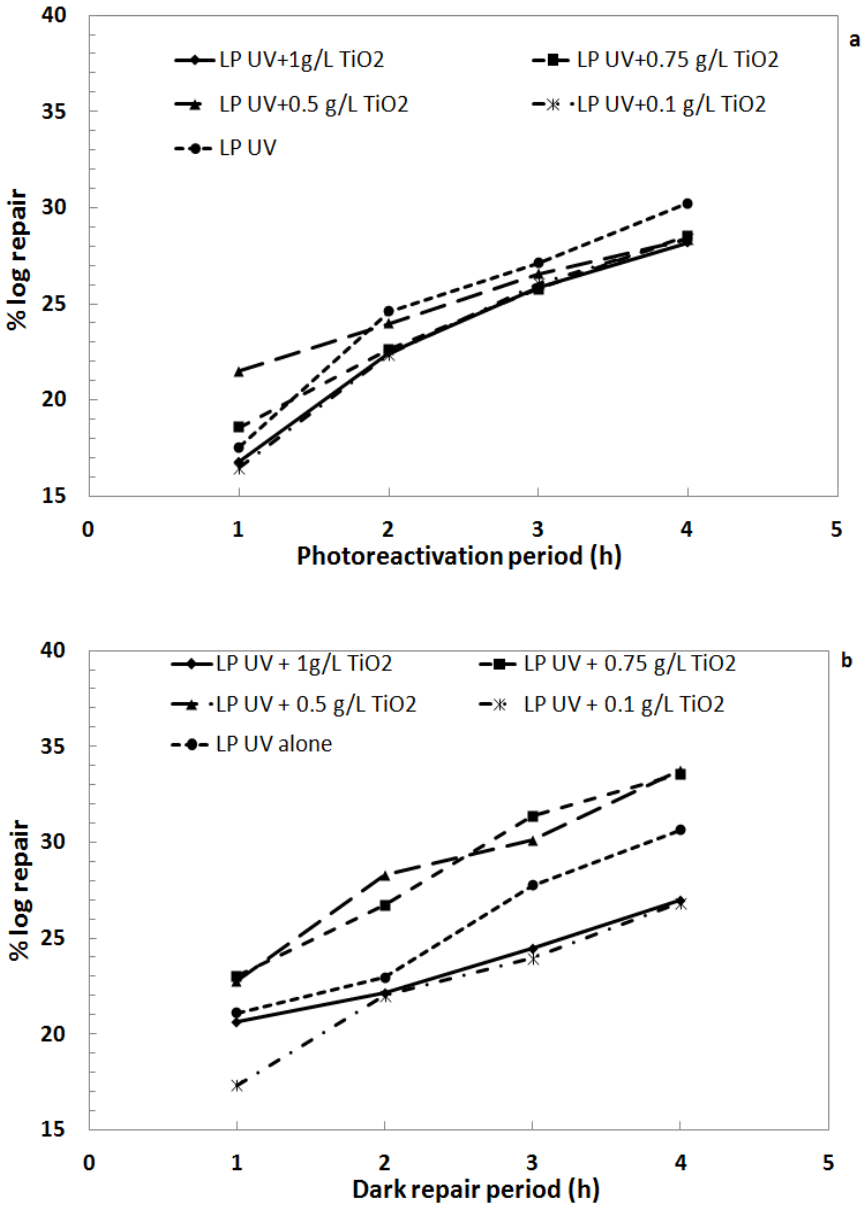


Figure 2.2 Percent log repair during 4 h repair period after LP UV and LP UV-TiO_2 (different concentrations) irradiation: (a) shows photoreactivation period and (b) shows repair during dark period.

Photocatalysis might have caused oxidation of photolyase enzyme which is responsible for repairing pyrimidine dimers back to original state. This was achieved through the oxidative damage done to the cellular compounds and cell membrane, which had lead cells in a condition beyond which they could repair by photoreactivation (Dunlop *et al*. 2002; Benabbou *et al*. 2007; Shang *et al*. 2009; Chong *et al*. 2010). It should be noted that Liu and Yang (2003) have found that higher TiO_2 concentration had resulted in a significant log reduction. Lose of cultivability was observed after disinfection under LP UV and photocatalysis, due to reduction in colonies forming units on agar plates.

No reactivation in dark was observed by Benabbou *et al*. (2007) after UV-C and photocatalyis using TiO_2 at varying concentrations. However, we had observed significant recovery of *E. coli* during photoreactivation and dark repair. It could be seen that *E. coli* cells were being repaired even after 2 h of dark repair (Figure 2.2b). This finding is not in agreement with previous study (Quek and Hu 2008) in which dark repair was leveled off beyond 2 h of repair after reaching maximum value. Although, a gradual leveling off was seen for photoreactivation period beyond 2 h which is in agreement with previous study (Quek and Hu 2008). This shows that addition of TiO_2 had lowered the number of bacterial cells which could repair their damaged DNA and pyrimidine dimers under visible light conditions. The discrepancies in results could be attributed to difference in *E. coli* strains used, variation in experimental geometry, and difference in UV intensities utilized for irradiation.

2.4.2 *E. coli* with intact cell membrane after LP UV and LP UV-TiO_2 disinfection: flow cytometry

All water samples were analyzed by flow cytometer to enumerate cells with or without cell membrane damage, as assessed by their ability to exclude propidium iodide (Berney *et al*. 2007; Hammes *et al*. 2008; Khan *et al*. 2010; Remy *et al.* 2012). Baclight® kit contains nucleic acid stains which compete for the same target location when both stains are present. STYO9 binds to the nucleic acid of microorganisms irrespective of the cell membrane condition, whereas propidium iodide (PI) only enters cells with damaged cell membrane and stains nucleic acid. The standard side scatter plot was generated for *E. coli* cells treated with 0.85% saline solution (intact cell membrane, live cells) and 70% isopropyl alcohol (compromised cell membrane, dead cells) is shown in figure 2.3. The standard side scatter plot shows only two main regions, namely: live and dead region for intact cell membrane and compromised cell membrane, respectively.

Whereas, side scatter plot for all the treatments (Figure 2.4) shows three states of *E. coli* namely: intact cell membrane (live), compromised cell membrane (dead), and intermediate cells after 4 h of photoreactivation and dark repair. It is evident from flow cytometry analysis that LP UV-TiO_2 treatments had induced an intermediate state between *E. coli* with compromised (dead) and intact (live) cell membrane. The intermediate region might have resulted from the partial oxidation of cell membrane by reactive oxygen species, thereby leading to a state where SYTO9 and PI had equal competence for DNA (Figure 2.4). The detection of intermediate state with flow

cytometry using variable amount of stains have also been reported in previous research (Berney *et al*. 2008; Khan *et al*. 2010).

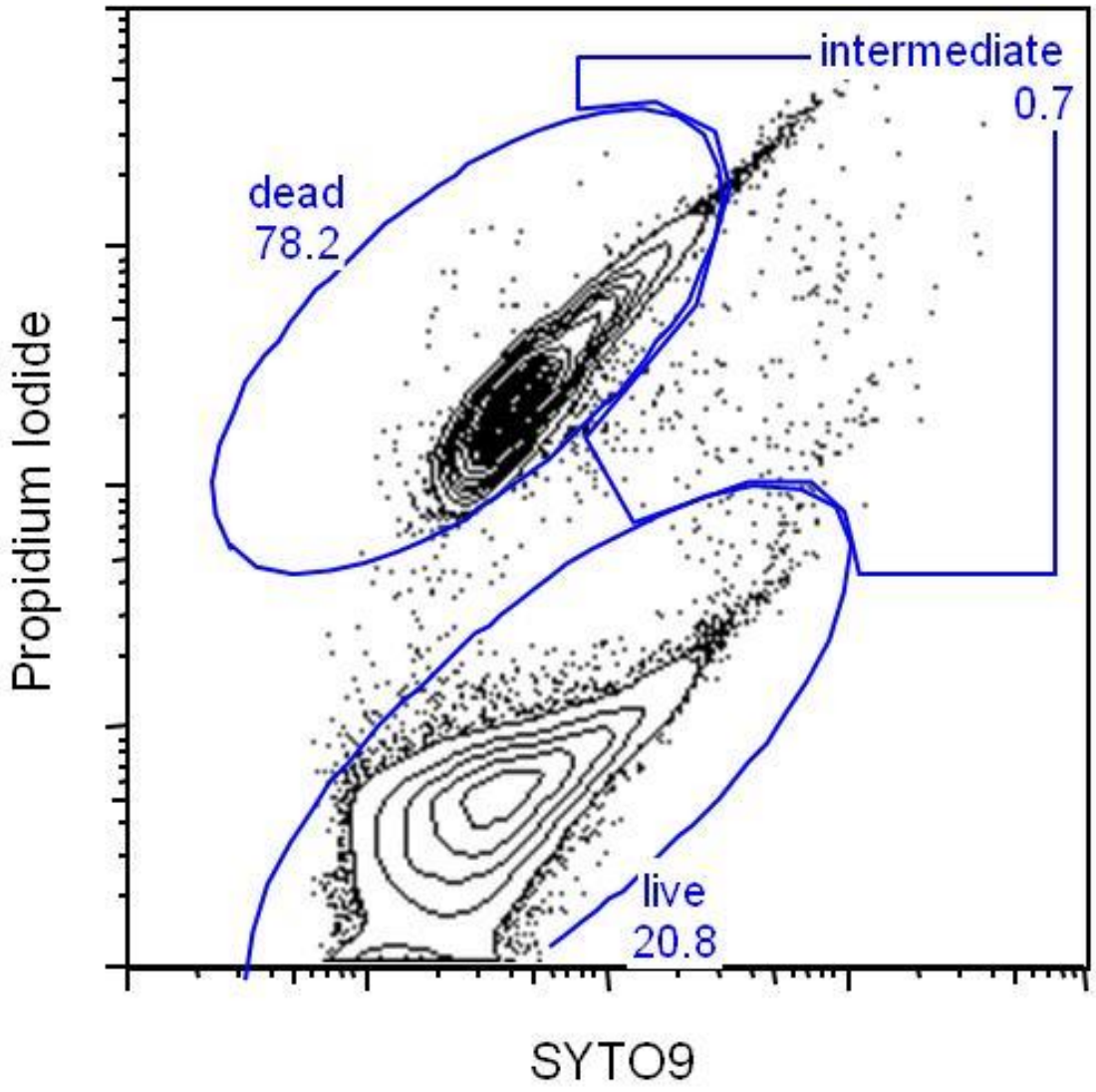


Figure 2.3 Standard side scatter plot for *E. coli* treated with 0.85% saline solution (intact cell membrane) and 70% isopropyl alcohol (compromised cell membrane) as determined by flow cytometry.

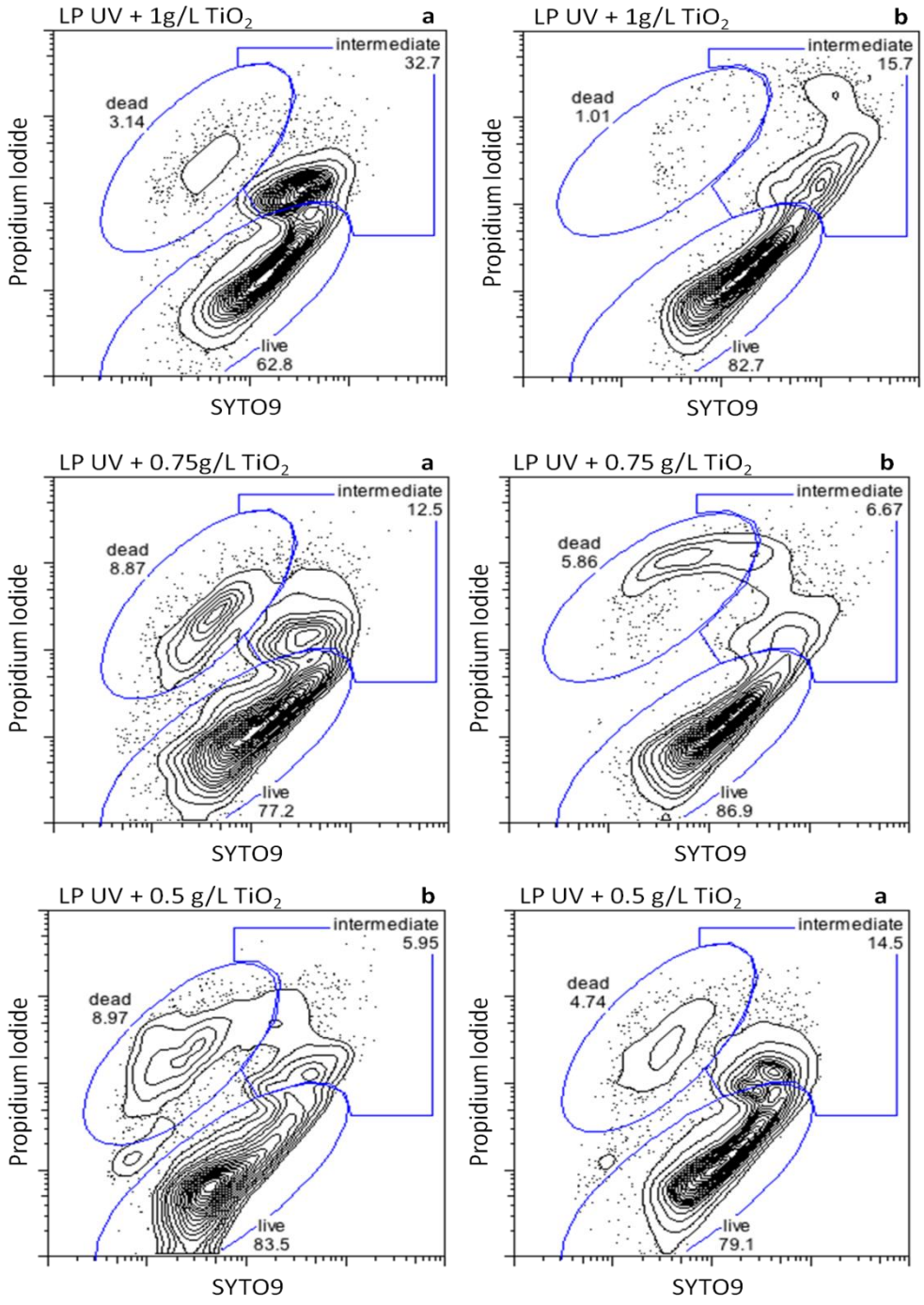


Figure 2.4 Side scatter plot of *E. coli* with intact and compromised cell membrane after 1 h of photoreactivation (a) and dark repair (b) for all the treatments as determined by flow cytometry.

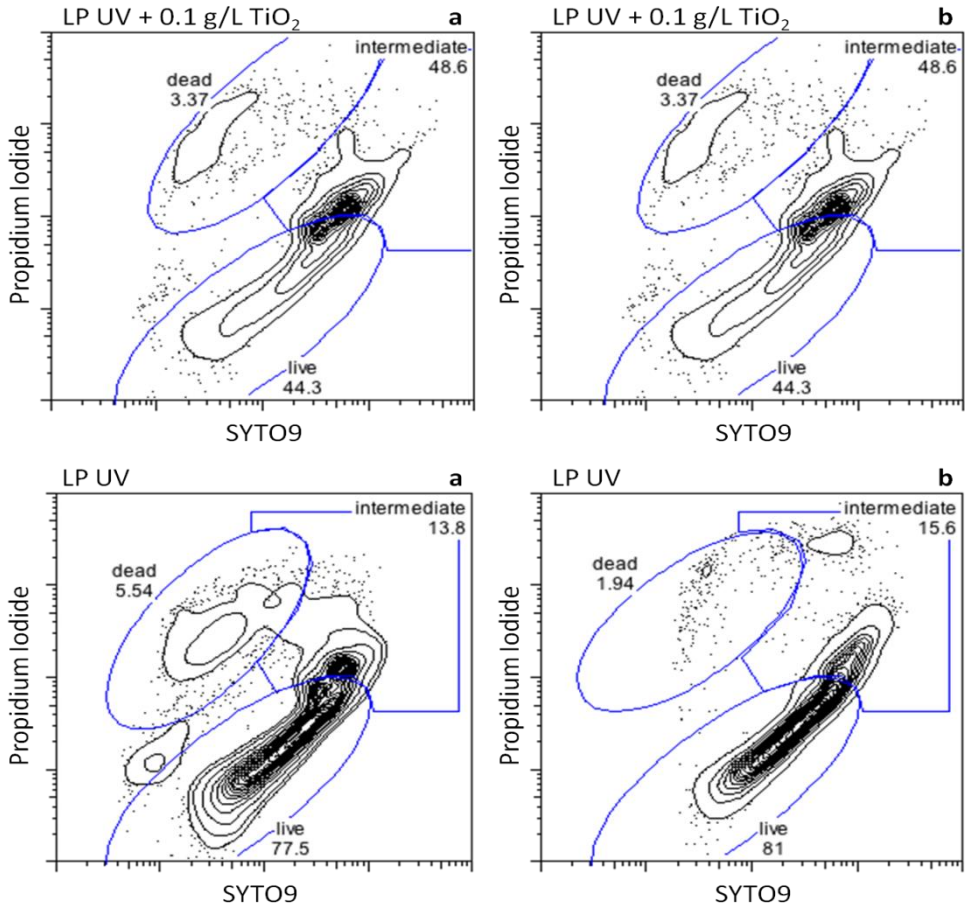


Figure 2.4 Continued.

E. coli with intact cell membrane counts during photoreactivation and dark repair for all the treatments are shown in figure 2.5. The initial *E. coli* intact cell membrane concentration was 10^9 counts/mL and after disinfection, minor log reduction in concentration was seen. It is in contrast to the CFU/mL obtained from culture based method, which showed a 5 $\log_{10}$ reduction after disinfection. During the repair period, flow cytometry showed 4-5 $\log_{10}$ higher counts than culture based method. Flow cytometry detection is irrespective of the culturability of the cells, and this finding is also supported by previous research (Egli 2008; Hammes *et al*. 2008; Khan *et al*. 2010). It could be seen that there was not extensive damage done to the cell membrane due to reactive oxygen species, such as hydroxyl radicals. The results are contrary to a previous study which had reported that cell membrane integrity was lost after UV-C and TiO_2 based photocatalysis (Gogniat *et al*. 2006; Remy *et al.* 2012). They had utilized LIVE/DEAD Baclight kit and assessed the membrane damage using fluorescence microscope. Their results had indicated the cell membrane integrity was lost after exposure to UV-C irradiation and under photocatalysis. UV-C irradiations not only damages the DNA but also gets absorbed by membrane amino acids and lipids, which gets oxidized, and results in cell membrane damage (Remy *et al.* 2012).

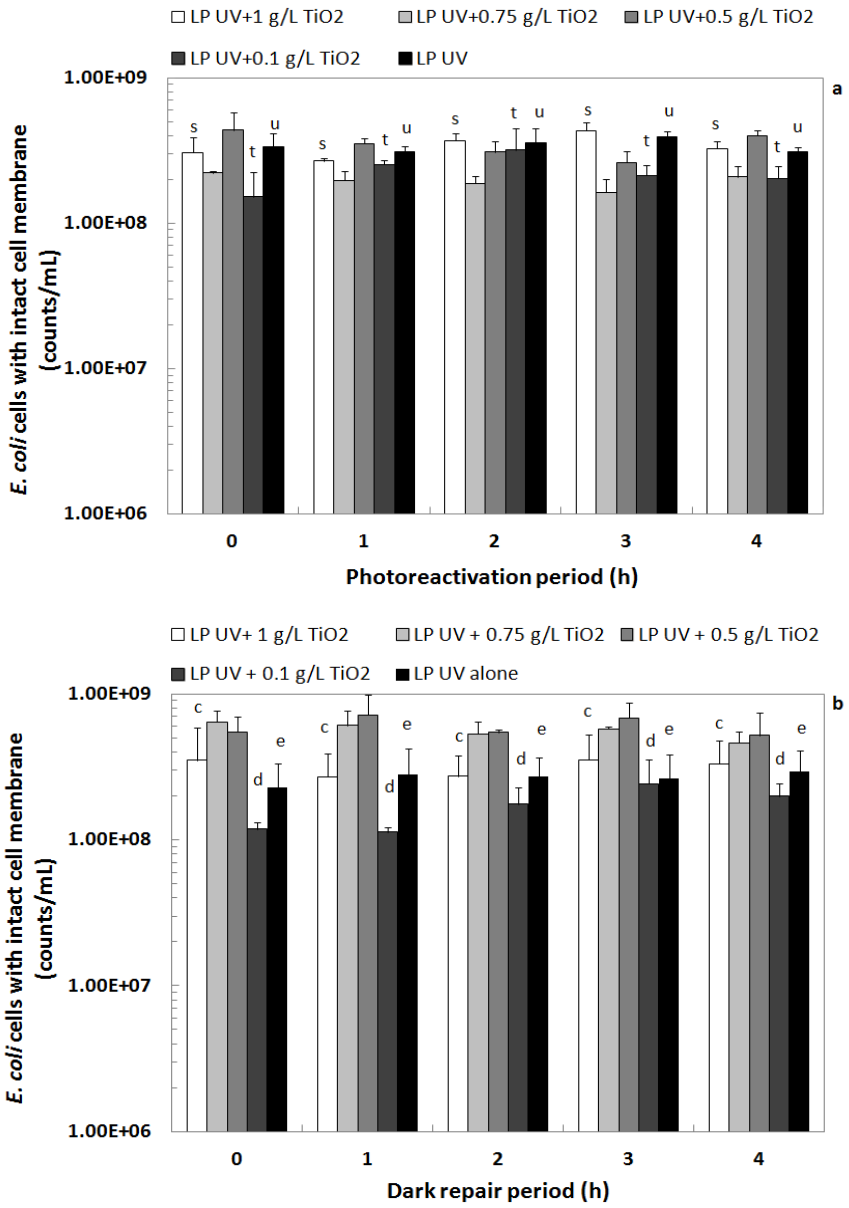


Figure 2.5 *E. coli* cells with intact cell membrane (viable cells) during 4 h repair period after LP UV and LP UV-TiO_2 disinfection as determined by flow cytometry: (a) shows photoreactivation period (*s* and *u* are not significantly different, $p = 0.9118$; *t* and *u* are significantly different, $p = 0.0002$) and (b) shows repair during dark period (*c* and *e* are not significantly different, $p = 0.2165$; *d* and *e* are significantly different, $p < 0.0001$). Standard deviations of three experiments are presented as error bars.

However, in this study more than 80% cells had intact membrane for all the treatments, except for the treatment with TiO_2 at 0.1 g/L concentration, which had only 50% of cells with intact membrane. It could be seen that LP UV+0.1 g/L TiO_2 treatment had resulted in lower intact cell membrane counts in comparison to the remaining treatments. It is in agreement with the finding from the culture based method in our study (Figure 2.1). However, the intact cell counts measured by membrane integrity were higher than culturable counts, implying presence of VBNC state cells. However, Gogniat *et al*. (2006) had found no VBNC state cells after TiO_2 based photocatalysis. It can be speculated that reduction seen in *E. coli* numbers could be due to DNA damage, some cell membrane damage, and possible reduction in metabolic activity.

While determining the water quality of highly sensitive distribution areas such as hospitals, nursing homes, healthcare facilities, and pharmaceutical and food manufacturing industries culture based methods will underestimate the concentration of pathogens, while flow cytometry will provide more realistic numbers (Egli 2008; Hammes *et al*. 2008; Khan *et al*. 2010). Furthermore, culture based methods cannot detect viable but non culturable (VBNC) cells. VBNC is that state of bacteria which is related to the inability of the microorganism to recover metabolic activity and is considered to be reversible, as cells could be revived to culturable state, once they are exposed to favorable growth conditions. Cells retain their intact cytoplasmic membrane even when they had lost cultivability (Oliver 2010). Also, microorganisms might produce enterotoxins and remain virulent in VBNC state (Khan *et al*. 2010; Oliver

2010). To avoid potential health risk, further improvement in the water disinfection systems will be required to maintain an effective level of pathogen reduction.

2.4.3 Effects of photocatalysis (LP UV-TiO_2) on repair of *E. coli*

The *E. coli* concentrations measured in CFU/mL from culture based and cell counts/mL from flow cytometry methods followed normal distribution as determined from the normality plot of residual. The data also satisfied the conditions of randomness and equal variance as observed from residual *vs.* predicted and Box Cox plots, respectively (data not shown). The culturable (determined from culture based method) and intact cell membrane counts (determined from flow cytometry) were significantly different for all the treatments ($p < 0.0001$, Table 2.2). The mean culturable *E. coli* cell counts were significantly affected by the titanium dioxide concentration. Cell counts during photoreactivation and dark repair showed a significant difference among five treatments (LP UV-TiO_2 and LP UV) for both culture based and flow cytometry methods ($p < 0.0001$, Table 2.2).

Photoreactivated and dark repaired cell counts for LP UV were significantly higher ($p < 0.0072$, $p < 0.0001$, Figure 2.1) than LP UV + 1 g/L TiO_2 as determined by culture based method, but were not statistically different for flow cytometry methods ($p = 0.9118$, $p = 0.2165$, Figure 2.5). On the other hand, photoreactivated cells for LP UV + 0.1 g/L TiO_2 were significantly lower than LP UV treatment for both culture based ($p < 0.0001$, Figure 2.1) and flow cytometry methods ($p = 0.0002$, $p < 0.0001$, Figure 2.2).

Table 2.2 Two way ANOVA analysis on culture based and flow cytometry data for (a) photoreactivation and (b) dark repair

Source	Culture based method				Flow cytometry method			
	F value[a]	**p-value[a]**	**F value[b]**	**p-value[b]**	**F value[a]**	**p-value[a]**	**F value[b]**	**p-value[b]**
Model	819.61	<0.0001	175.6	<0.0001	9.11	<0.0001	43.13	<0.0001
A-TiO_2	20.34	<0.0001	30.65	<0.0001	17.81	<0.0001	84.89	<0.0001
B-Time (h)	1618.88	<0.0001	320.55	<0.0001	0.40	0.8060	1.37	0.2450

[a]photoreactivation.
[b]dark repair.

Thus, statistically it was evident that addition of photocatalyst at 0.1 g/L along with LP UV had resulted in significant difference in cell counts and reduced the subsequent percentage repair after disinfection. It could be deduced that dark repair percentages could be significantly reduced by adding TiO_2 at 0.1 g/L to the UV disinfection systems, as compared to LP UV alone disinfection system. Previous studies have shown that photocatalysts play an important role in inactivation of pathogenic microorganisms present in water (Dunlop *et al*. 2002; Liu and Yang 2003; Shang *et al*. 2009). This study not only agrees with previous findings but also showed that photocatalyst enables in curbing the dark repair of *E. coli* after photocatalysis under LP UV. Modification of the disinfection treatment should be done by accounting for repair rates of microorganism in water. While considering the effectiveness of a water disinfection system, reactivation after disinfection should be evaluated. This study provides direct evidence that repair of *E. coli* is possible after disinfection.

Flow cytometry had showed significantly higher cell counts than culture based method. The culture based method had estimated only a fraction of the total bacterial cells present in water in contrast to detection using flow cytometry and nucleic acid dye stain. The number of viable pathogenic cells as enumerated by widely used culture based method will be less accurate in accessing the microbial quality of drinking water (Berney *et al*. 2007; Egli 2008; Hammes *et al*. 2008; Khan *et al*. 2010). Culture based methods underestimate cell numbers due to (1) cell injury, (2) inability to take up nutrients in the medium, and (3) other physiological factors which hampers culturability (Khan *et al*.

2010). Bacterial repair and regrowth is also affected by presence of inhibitors in selective media (Dunlop *et al*. 2002).

While assessing disinfection systems, total bacterial cell counts, rapid detection of pathogens, and microbial viability should be evaluated (Egli 2008). On average 60-90% microorganisms present in water are biochemically active and significantly higher than detected by cultured based methods (Egli 2008; Hammes *et al*. 2008). Furthermore, intermediate states like cell membrane damage, cell injury are difficult to detect using culture based methods. So, for bacterial enumeration there is a need for fast, reliable and accurate method which could be utilized in optimizing water disinfection system. The repair of bacteria should be considered for the time involved between the disinfection system and final end use points. It is very critical for selecting appropriate retention time in water distribution systems after disinfection.

CHAPTER III

PHOTOREACTIVATION AND DARK REPAIR OF ENVIRONMENTAL *E. COLI* STRAINS FOLLOWING 24 kHz CONTINUOUS ULTRASOUND AND UV-C IRRADIATION

3.1 Overview

The effects of 24 kHz continuous ultrasound and UV-C on inactivation and potential repair of environmental *E. coli* strains were studied. Approximately 8 $\log_{10}$ reduction was observed after 15 and 20 min of ultrasound and UV-C application, respectively. Inactivation rate of 0.53 log min^{-1} was observed for ultrasound disinfection. AlamarBlue® assay was used to assess the metabolic activity of *E. coli* cells during the inactivation and repair period. The metabolic activity of *E. coli* cells after ultrasound disinfection was greatly reduced than after UV-C disinfection. Dark repair and photoreactivation of *E. coli* strains were studied over a 24 h period. *E. coli* isolate from treated wastewater effluent had repaired nearly 53% and 82% (24 h photoreactivation conditions) after ultrasound and UV-C treatment, respectively. Laboratory *E. coli* strain had photo-reactivated by 30% and 42% after ultrasound and UV-C disinfection, respectively. Possible shearing effect and reduction in cell size of *E. coli* exposed to ultrasound was revealed by transmission electron micrographs. The results suggest that ultrasound could be used as an effective means of disinfecting *E. coli* cells by shearing and decreasing repair after UV-C and ultrasound.

3.2 Introduction

Inactivation of pathogenic microorganisms is the goal of water disinfection processes. Variety of chemical and physical disinfection processes such as chlorination, ozonation, photocatalysis, and ultraviolet light (UV-C) disinfection have been extensively used in water treatment. Physical treatments have gained popularity over chemical processes, as latter suffer from the formation of disinfection by-products, which are carcinogenic in nature (Boorman *et al*. 1999; Hua and Thompson 2000; Liu and Yang 2003; Galvez *et al*. 2007; Quek and Hu 2008; Drakopoulou *et al*. 2009). Efficiency of physical methods like UV disinfection depends upon light absorbing and scattering particles and repair potential of microorganisms (Drakopoulou *et al*. 2009). Furthermore, microorganisms are becoming resistant to many existing disinfection applications, such as chlorination, and UV irradiation (Furuta *et al*. 2004). Effective and alternative treatments are necessary for disinfecting water.

Ultrasound irradiation has been used for water disinfection. Mechanical vibrations of frequencies between 20 kHz and 800 kHz result in generation of ultrasound waves. Sonication involves generation of microbubbles through ultrasound waves to create oxidative environment via cavitation (Paleologou *et al*. 2007). Underlying principle of sonication lies in acoustic cavitation, in which microbubbles are formed and collapsed in milliseconds (Hua and Thompson 2000; Drakopoulou *et al*. 2009). Thus, extreme temperature and pressure gradients are generated on collapse of these microbubbles, which result in shear mechanical stress (Drakopoulou *et al*. 2009). These short-lived conditions are in turn responsible for generation of highly reactive radicals

which are accountable for oxidative damage (Paleologou *et al*. 2007; Drakopoulou *et al*. 2009). High power ultrasound at low frequencies (20 kHz to 100 kHz) is an effective means for disintegrating bacterial cells due to its ability to cause cavitation (Piyasena *et al*. 2003; Cameron *et al*. 2008).

Disinfection treatment efficiency depends upon many factors such as solution temperature, turbidity, and microorganism's resistance to inactivation. Hua and Thompson (2000) had investigated practicality of ultrasound irradiation as a physical-chemical method for disinfection of *E. coli* in water. They had investigated the effects of ultrasound frequency, dissolved gas, and acoustic power intensity on the inactivation of *E. coli* during sonolysis (Hua and Thompson 2000). For water/wastewater disinfection, combination of ultrasound with UV, hydrogen peroxide, chlorine, and ozone have been demonstrated in many recent studies (Hua and Thompson 2000; Madge and Jensen 2002; Paleologou *et al*. 2007). However, the main focus of disinfection studies was on pure/reference strains of bacteria or yeast, rather than on environmental strains. Ultrasound disinfection has been studied on a range of reference strains of bacteria, but not much work has been done on evaluating disinfection treatments on environmental strains. To effectively determine cell viability after disinfection treatment, utilization of molecular assays should be incorporated to conventional culture based methods. Determination of cell viability and membrane oxidation is limited by conventional culture based method.

In this study, inactivation of three environmental *E. coli* strains isolated from fecal samples of feral hog and deer, and treated wastewater effluent from a wastewater

treatment plant (WWTP) was studied and compared with a laboratory *E. coli* strain (ATCC® 10798). Effects of 24 kHz continuous ultrasound and UV-C irradiation on inactivation and potential repair of four *E. coli* isolates through a culture based method and a metabolic activity assay.

3.3 Materials and Methods

3.3.1 Bacterial strains

Four *E. coli* isolates were selected for inactivation and potential repair studies. Laboratory *E. coli* K-12 strain was obtained from American Type Culture Collection (ATCC®10798); and the remaining three isolates were obtained from wastewater treatment plant (WWTP) effluent and fecal samples of feral hog and deer. All three environmental *E. coli* samples were collected from Attoyac Bayou watershed in Texas. Soil and Aquatic Microbiology Laboratory at Texas A&M University, College Station, TX has performed the bacterial isolation.

E. coli culture was grown in Luria Bertani broth (LB broth) at 37°C for 24 h and was harvested by centrifugation at 10,000 × *g* for 3 min. The supernatant was removed and re-suspended in 0.85% saline solution and re-centrifuged. The pellet was washed twice to remove the nutrients. The initial concentration of washed *E. coli* was maintained at 10^8 cells/mL and diluted 10 times in the batch reactor, described in the following sections. All glassware, nuclease free deionized (DI) water and media used for bacterial growth were autoclaved prior to each experiment.

3.3.2 Disinfection treatments

Ultrasonication

Ultrasound irradiation was provided by the horn type sonicator, operating at 24 kHz and 400 W (UP400S Hielscher, Germany). The experiment was conducted in triplicates in 50 mL BD Falcon® centrifuge tubes. The working volume of the batch reactor was 40 mL, which included 0.85% saline solution and nuclease free DI water. The sonotrode was aligned to the center of the BD Falcon® centrifuge tube. The distance between the tip of the sonotrode and bottom of the centrifuge tube was set at 1 cm. The temperature of the reactor was not controlled in order to study the combined effect of sonication and heat on inactivation of *E. coli*. The temperature in the batch reactor was allowed to reach up to 65±5°C. The ultrasound power received by the *E. coli* suspension in batch reactor at 100% amplitude and cycle one was 100 W, as estimated by calorimetric measurement. Calorimetric measurement was performed by recording water temperature at specific time intervals under ultrasound application in an insulated container. Energy required to heat up the water for a particular time interval was calculated using the specific heat capacity of water. The surface area of the horn type sonotrode H3 was 0.0706 cm^2 and the ultrasound intensity applied was 1416 W/cm^2 (ratio of power to sonotrode surface area). To accurately study the reactivation of microbial cells, complete inactivation was achieved to eliminate regrowth. The batch reactors were subjected to ultrasound to achieve microbial inactivation close to detection limit of 10 CFU/mL (i.e., for 100 μL plated on agar plates, at least one colony forming unit should be observed). A control sample (with cells but not exposed to ultrasound)

served as positive control. For negative control, nuclease free DI water with no cells was used. The horn type sonicator was stopped after required irradiation and complete kill. The batch reactors were then exposed to visible light with an average intensity of 30,000 lux for photoreactivation to occur for 24 h. For dark repair, batch reactors were covered with aluminum foil for 24 h. All the experiments were performed once in triplicate at an average pH 7.0±0.5. Regular time interval samples were taken during repair period at time t = 1, 2, 7, and 24 h.

Ultraviolet irradiation

A UV-C low pressure (LP) germicidal lamp (Bryant energy, Indianapolis, USA) with wavelength of 254 nm was mounted horizontally in a polyvinyl chloride (PVC) box with dimensions of 81 × 61 × 51cm (L × B × H). The distance between UV lamp and water surface was kept at 12 cm. The average UV irradiance measured before sample exposure using a radiometer ranged from 3.25 to 3.27 mW/cm^2 (I_{avg}). The effect of water depth of 1 cm on absorbance of UV irradiance was neglected. The required UV dose was calculated using the following equation by adjusting the exposure time to achieve inactivation close to detection limit (10 CFU/mL) for all the treatments (Hallmich and Gehr 2010).

$$\text{UV dose (mW. s/cm}^2\text{)} = I_{avg}\,(\text{mW/cm}^2) \times \text{time (s)} \quad (3.1)$$

The experiment was conducted once in triplicate in a 200 mL Pyrex® beakers at an average pH 7.0±0.5 and 22±1ºC to ensure their reproducibility. The 40 mL working volume contained autoclaved DI water and 0.85% saline solution. The batch reactors were subjected to constant mixing using the magnetic stir plate at 200 rpm and were

irradiated under UV-C lamp to achieve kill close to detection limit (10 CFU/mL). The whole setup was covered in a PVC box to cut off ambient light. A control sample without cells served as negative control. Another control sample (with cells but not exposed to irradiation) was also utilized during the experiments. After the required irradiation period, photoreactivation and dark repair were studied. The batch reactors were exposed to visible light for photoreactivation to occur for 24 h. Similarly for dark repair study after disinfection, batch reactors were covered with aluminum foil for 24 h. Regular time interval samples were taken during repair period at time t = 1, 2, 7, and 24 h.

3.3.3 *E. coli* cell enumeration and metabolic activity

Culture based method - plating

E. coli concentrations were determined by the spread plate method. At regular time interval sampling, triplicate plates were inoculated for each batch reactors. For this method, appropriate dilutions were performed in de-ionized nuclease free water and 100 µL of the diluted sample was spread on the MacConkey agar plates. The plates were then incubated for 24 h at 37±1°C. After incubation, colonies were enumerated as CFU/mL and average CFU/mL for all the triplicate experiments was recorded.

Cell viability assay

Cell viability and metabolic activity of *E. coli* were assessed by the AlamarBlue® cell viability assay. A 10X, AlamarBlue® cell viability reagent was used to assess the cell viability. Only 10% of the AlamarBlue® reagent, i.e., 10 µL was added to 100 µL of bacterial cells according to the manufacturer's assay protocol (Invitrogen,

USA). The reagent was used in a 96 well plate format in a "no wash" mode. The 96 well plate was then incubated at 37°C for desired time to allow reduction of resazurin to resorufin. Samples with high cell counts were incubated for 2 h, whereas samples with low cell counts after disinfection were incubated for 24 h, as per manufacturer's protocol. AlamarBlue® added to medium without cells served as the negative control. After the desired incubation, the resulting fluorescence was read using a Victor 2 multi-label counter 1420 microplate reader at 560 nm (Wallac 1420 Victor 2, Perkin Elmer, Massachusetts, USA). The resulting fluorescence value from the negative control was subtracted from the fluorescence reading for each sample.

3.3.4 Transmission electron microscopy (TEM)

Before and after disinfection treatments, 1 mL of *E. coli* was extracted and centrifuged at 10,000 × *g* for 5 min. The supernatant was discarded and the pellet was re-suspended in 0.85% saline solution and re-centrifuged. The extracted cells were then provided to the Microscopy Imaging Center at Texas A&M University, College Station, TX. Transmission electron microscope (JEOL 1200ex) was utilized for imaging *E. coli* cells by following standard protocol for transmission electron microscopy.

3.3.5 Repair assessment

In order to assess photoreactivation and dark repair of *E. coli*, two parameters, namely: percent log repair and repair rate were examined. To negate the variability in initial cell counts and log reduction after treatment, the following formulae were used (Quek and Hu 2008):

$$\% \text{ log repair} = \frac{(N_{initial} - N_0)}{(N_t - N_0)} \times 100 \quad (3.2)$$

Where N_t is log CFU/mL of *E. coli* at time of exposure, t, to photoreactivation/dark repair conditions; N_o is log CFU/mL of *E. coli* immediately after low pressure UV-C and ultrasound disinfection; and $N_{initial}$ is the initial concentration of *E. coli* before disinfection treatment (log CFU/mL). The rate of repair was calculated using the following equation (Quek and Hu 2008):

$$\text{Rate of repair } \left(\log h^{-1}\right) = \frac{(N_t - N_0)}{t} \quad (3.3)$$

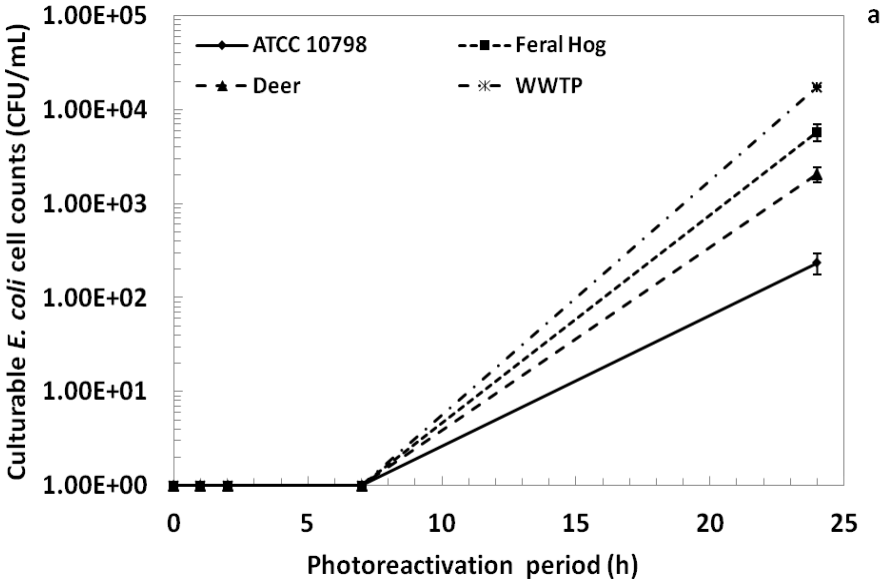
Where N_t is log CFU/mL of *E. coli* after "t" h of photoreactivation/dark repair and t is time interval between two samples. The rate of repair was studied after 24 h of disinfection.

3.4 Results

3.4.1 Culturable *E. coli* cells after ultrasound and UV-C disinfection treatments

The average initial concentration of *E. coli* ($N_{initial}$) was 10^7 CFU/mL. The ultrasound and UV-C disinfection had resulted in nearly 8 $\log_{10}$ reduction for all *E. coli* strains after 15 and 20 min of exposure, respectively. This disinfection was considered complete inactivation of *E. coli* cells. The inactivation occurred at a rate of 0.52 log min^{-1} and 0.39 log min^{-1} for ultrasound and UV-C disinfection, respectively. Figure 3.1 shows 24 h of photoreactivation and dark repair of *E. coli* strains after ultrasound inactivation. Extent of photoreactivation of *E. coli* isolate from WWTP

effluent was the highest among other *E. coli* strains; 53% log repair (Figure 3.2) with a net reduction of 3.64 $\log_{10}$ (Table 3.1).



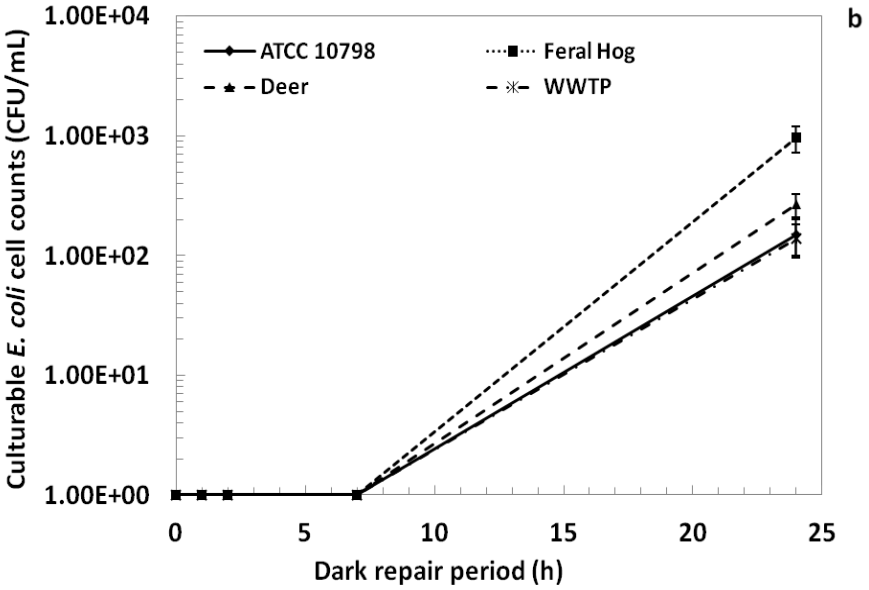
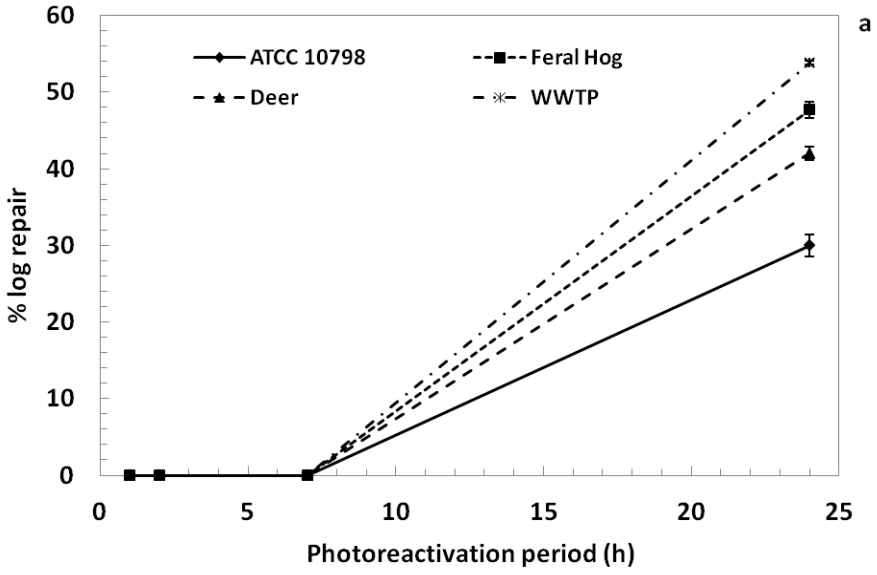


Figure 3.1 Culturable *E. coli* cell counts during 24 h of repair period after 24 kHz continuous ultrasound disinfection as determined by culture based method with a detection limit of 10 CFU/mL: (a) shows photoreactivation period and (b) shows repair during dark period. Standard deviation of three replicates is presented as error bar.



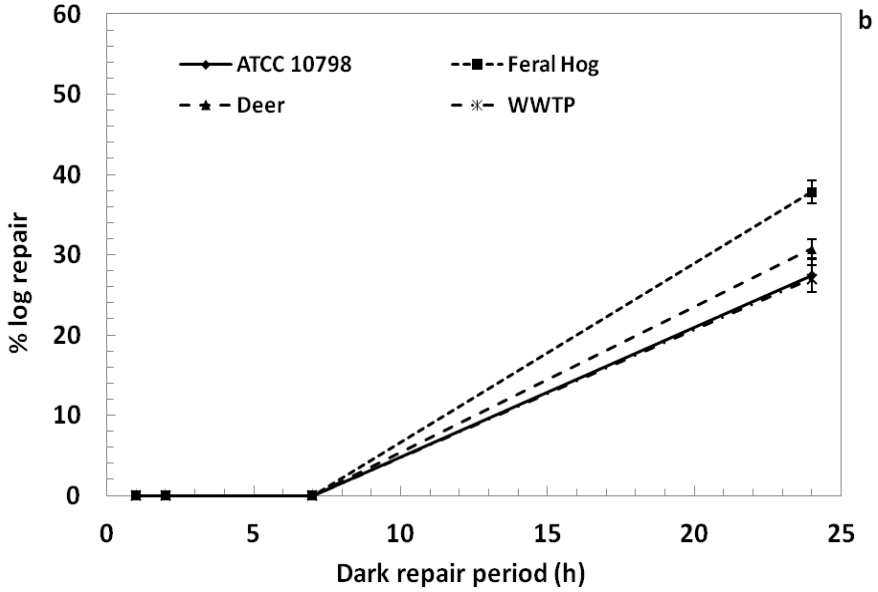


Figure 3.2 Percent log repair during 24 h repair period after 24 kHz continuous ultrasound disinfection with a detection limit of 10 CFU/mL: (a) shows photoreactivation period and (b) shows repair during dark period. Standard deviation of three replicates is presented as error bar.

Table 3.1 Culture based assessment of *E. coli* isolates after 24 kHz continuous ultrasound disinfection: (a) photoreactivation and (b) dark repair

	Net log reduction[1]		**% log repair**[‡]		**Repair rate (log h^{-1})**[‡]	
Bacteria	***a***	***b***	***a***	***b***	***a***	***b***
ATCC®10798	**5.51 ± 0.13**[§]	**5.71 ± 0.19**[§]	**29.98 ± 1.39**[§]	**27.44 ± 2.05**[§]	**0.10 ± 0.01**[§]	**0.09 ± 0.01**[§]
Feral Hog	**4.11 ± 0.08**[§]	**4.89 ± 0.12**[§]	**47.70 ± 1.08**[§]	**37.83 ± 1.40**[§]	**0.16 ± 0.02**[§]	**0.12 ± 0.02**[§]
Deer	**4.56 ± 0.06**[§]	**5.45 ± 0.09**[§]	**42.03 ± 0.91**[§]	**30.73 ± 1.14**[§]	**0.14 ± 0.01**[§]	**0.10 ± 0.01**[§]
WWTP	**3.64 ± 0.03**[§]	**5.75 ± 0.14**[§]	**53.81 ± 0.23**[§]	**27.02 ± 1.69**[§]	**0.18 ± 0.02**[§]	**0.09 ± 0.01**[§]

[1]Net log reduction over a 24 h repair period.
[‡]Twenty four hour repair period.
[§]Standard deviation of three replicates is presented.

E. coli isolate from feral hog feces had repaired the most (37%) during dark repair period with a net log reduction close to 5 $\log_{10}$ (Figure 3.2, Table 3.1). The percent log repair during photoreactivation was higher than dark repair for all *E. coli* isolates (Table 3.1). Laboratory *E. coli* strain (ATTC® 10798) had repaired the lowest under dark and photoreactivating conditions with an average log reduction of 5.6 $\log_{10}$. Until 7 h of repair, the percent log repair for all four *E. coli* isolates was close to zero (Figure 3.2). It was only 7 h after disinfection that an increase in *E. coli* concentrations was observed (Figure 3.1 and 3.2).

Figure 3.3 shows repair of *E. coli* isolates 24 h after UV-C disinfection. *E. coli* isolate from WWTP effluent had repaired the most under dark repair and photoreactivation periods with an average net log reduction of only 1.4 $\log_{10}$. The repair cell concentration for all the *E. coli* isolates until 7 h was $\leq$ 500 CFU/mL, with a maximum repair observed at 24 h. The average percent log repair for all *E. coli* isolates was nearly 30% util 7 h after disinfection (Figure 3.4). It could be evident that *E. coli* isolates had repaired by an average of 25% and 30% during first hour of photoreactivation and dark repair, respectively (Figure 3.4). The percent log repair was highest for *E. coli* isolate from WWTP effluent with nearly 81% for dark repair and 82% for photoreactivation (Table 3.2). Laboratory *E. coli* strain and isolate from feral hog feces were the lowest to repair under dark and photoreactivating conditions (Table 3.2).

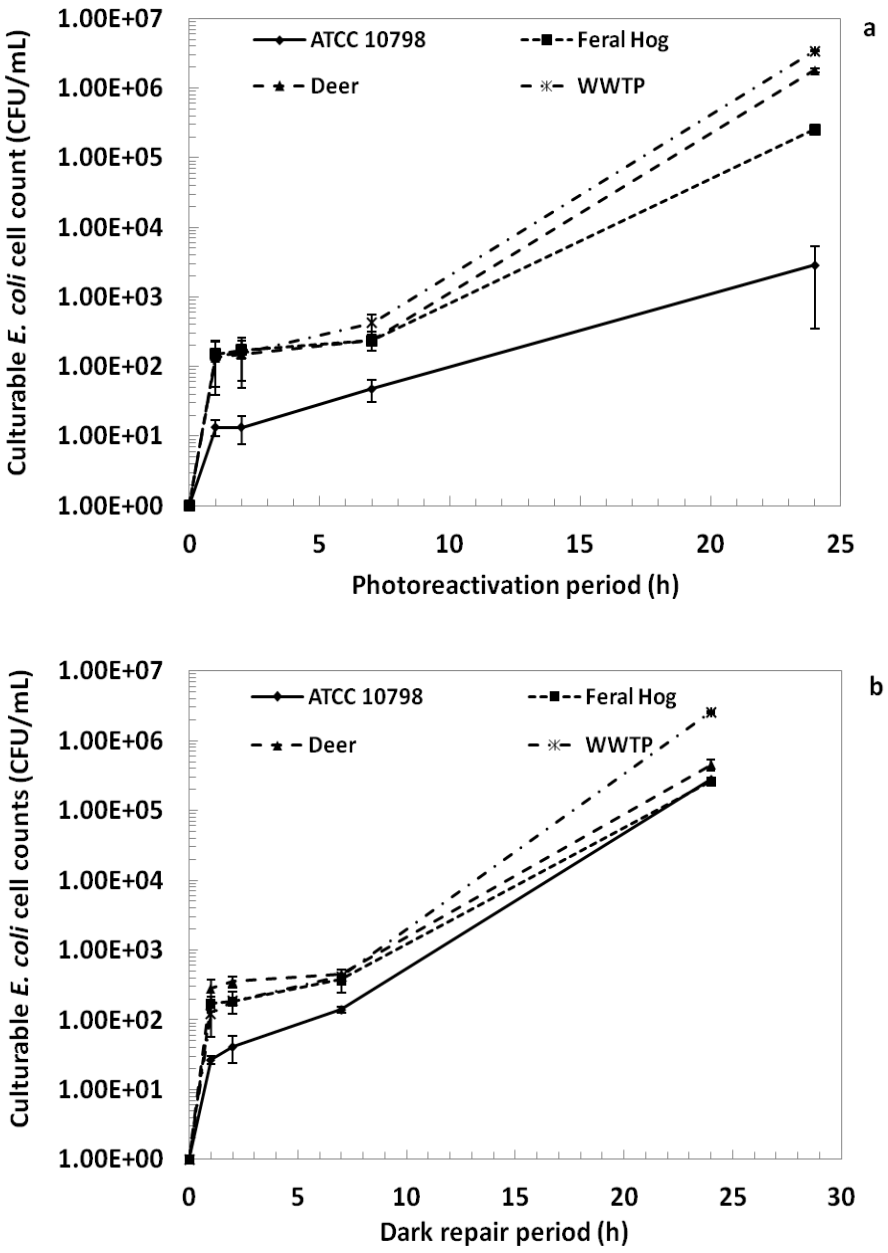


Figure 3.3 Culturable *E. coli* cell counts during 24 h of repair period after UV-C disinfection as determined by culture based method with a detection limit of 10 CFU/mL: (a) shows photoreactivation period and (b) shows repair during dark period. Standard deviation of three replicates is presented as error bar.

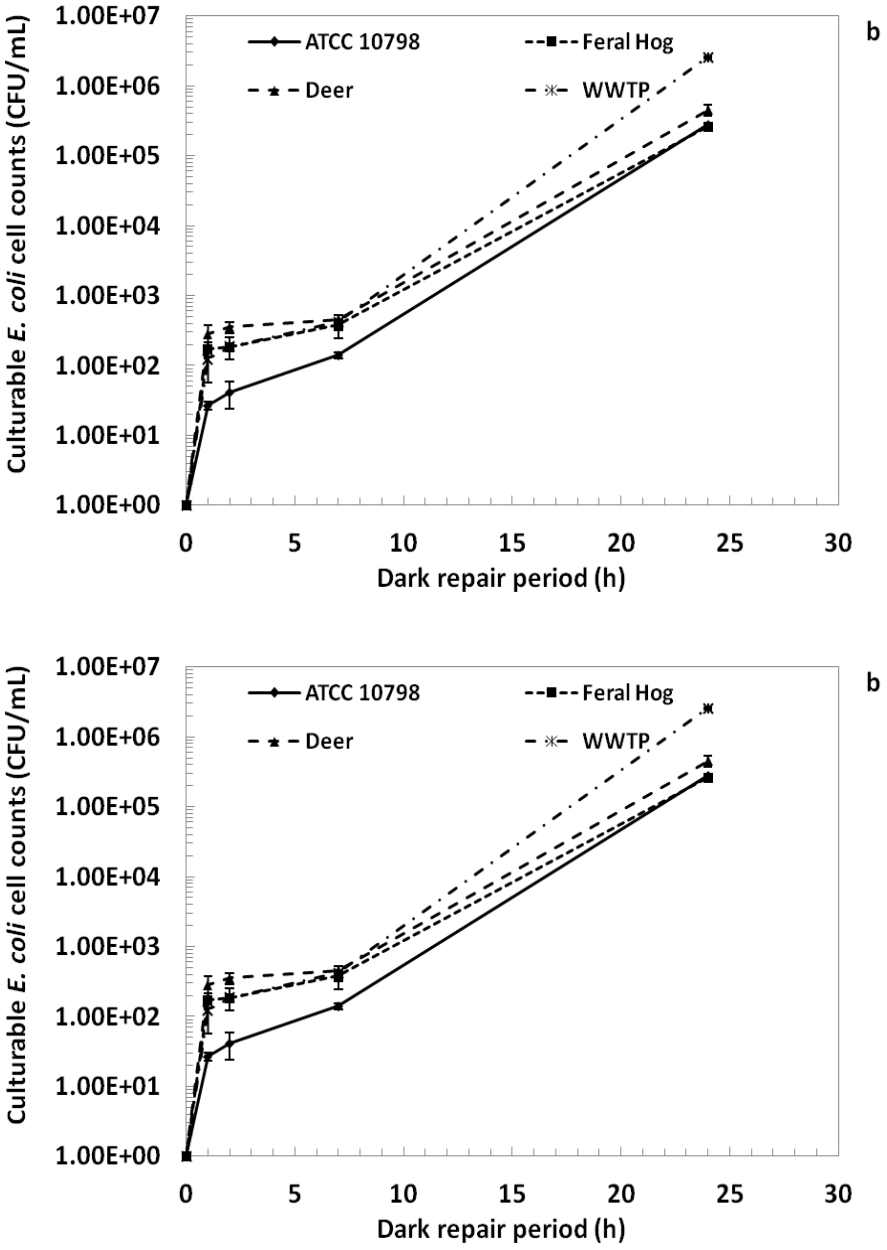


Figure 3.4 Percent log repair during 24 h repair period after UV-C disinfection with a detection limit of 10 CFU/mL: (a) shows photoreactivation period and (b) shows repair during dark period. Standard deviation of three replicates is presented as error bar.

Table 3.2 Culture based assessment of *E. coli* isolates after UV-C disinfection treatment: (a) photoreactivation and (b) dark repair

	Net log reduction [1]		**% log repair**[‡]		**Repair rate (log h^{-1})**[‡]	
Bacteria	***a***	***b***	***a***	***b***	***a***	***b***
ATCC® 10798	**4.53 ± 0.41**[§]	**2.42 ± 0.04**[§]	**42.52 ± 4.98**[§]	**69.20 ± 0.33**[§]	**0.14 ± 0.02**[§]	**0.23 ± 0.01**[§]
Feral Hog	**2.47 ± 0.06**[§]	**2.45 ± 0.01**[§]	**68.66 ± 0.67**[§]	**68.81 ± 0.12**[§]	**0.23 ± 0.01**[§]	**0.23 ± 0.02**[§]
Deer	**1.66 ± 0.05**[§]	**2.23 ± 0.08**[§]	**78.94 ± 0.68**[§]	**71.71 ± 1.06**[§]	**0.26 ± 0.01**[§]	**0.24 ± 0.01**[§]
WWTP	**1.35 ± 0.03**[§]	**1.47 ± 0.02**[§]	**82.89 ± 0.35**[§]	**81.29 ± 0.27**[§]	**0.27 ± 0.01**[§]	**0.27 ± 0.01**[§]

[1]Net log reduction over a 24 h repair period.
[‡]Twenty four hour repair period.
[§]Standard deviation of three replicates is presented.

3.4.2 Metabolic activity of *E. coli* isolates after disinfection treatments

The metabolic activity measured in terms of fluorescence @ 560 nm was around 130000 for healthy cells before disinfection. The metabolic activity of all four *E. coli* isolates after ultrasound disinfections was close to zero (data not shown) and comparable to negative control with no cells (fluorescence @ 560 nm was around 38000). Ultrasound disinfection had greatly affected the metabolic activity of the cells during the repair period. This observation is also in agreement with culturable cell count data shown in figure 3.1. The metabolic activity of all four *E. coli* isolates during repair period after UV-C disinfection treatments is presented in figure 3.5. Ultraviolet (UV) irradiation had less pronounced effect on the metabolic activity of the cells, as cells were able to maintain their activity immediately after the disinfection (fluorescence at t=0) and repair periods. In general, repair after 24 h was higher for *E. coli* cells exposed to UV-C than for cells exposed to ultrasound.

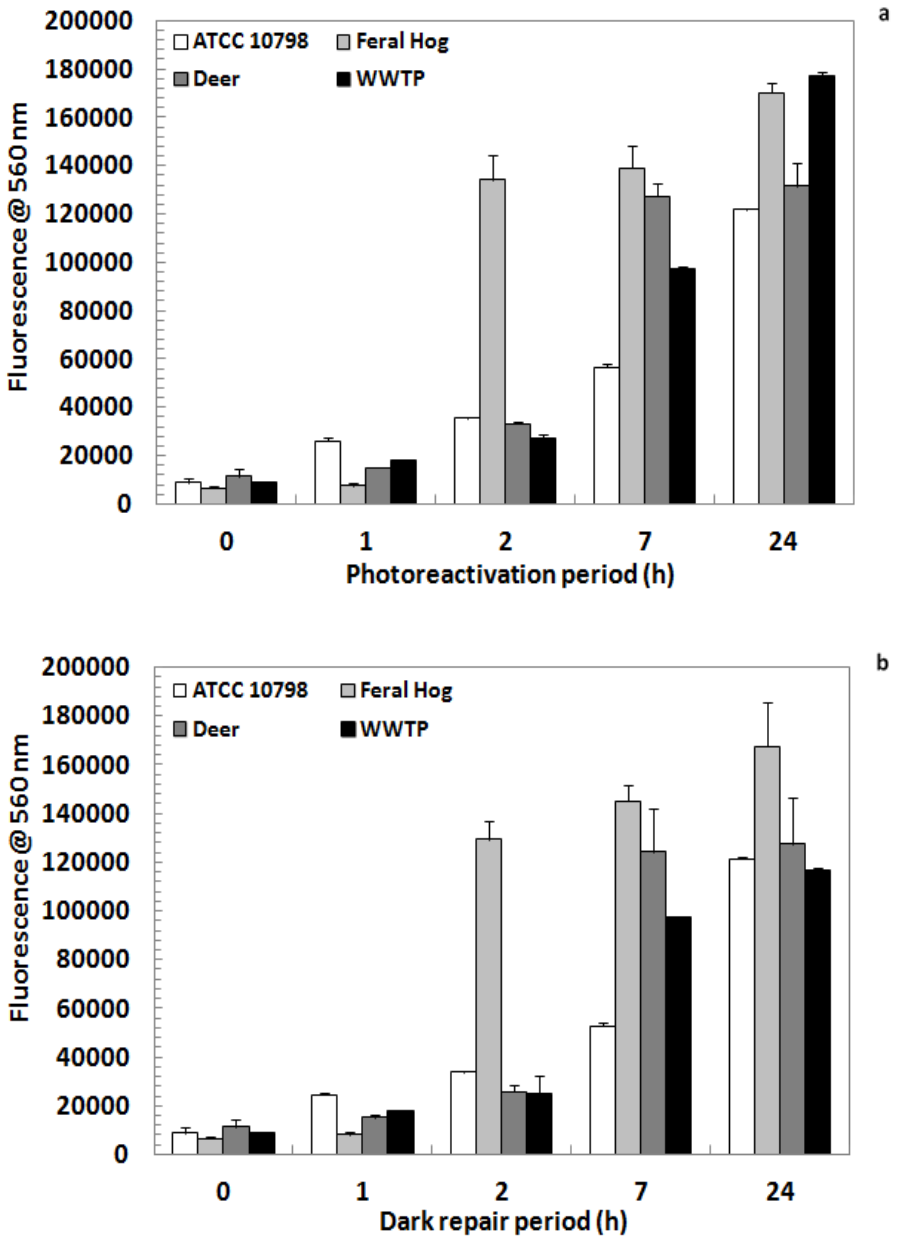


Figure 3.5 Metabolic activity of *E. coli* isolates as measured by AlamarBlue® fluorescence during 24 h of repair period after UV-C disinfection: (a) shows photoreactivation and (b) shows dark repair. Values were deducted from the fluorescence of AlamarBlue® without cells. Standard deviation of three replicates is presented as error bar.

3.4.3 Morphological changes in *E. coli* cells

Transmission electron microscopy (TEM) was utilized to investigate morphological changes in *E. coli* before (Figure 3.6a) and after exposure to 24 kHz ultrasound (Figure 3.6b) and UV-C irradiation (Figure 3.6c). The images were representative of samples under microscopic observation. It is evident that that *E. coli* cells were severely damaged by ultrasound (Figure 3.6b) which might be due to shearing of the cell wall and reduction in cell size. There was no significant changes to the cell wall for UV-C treated *E. coli* as seen under TEM (Figure 3.6c).

3.5 Discussion

In this study, temperature was not controlled during ultrasonication; and inactivation might be due to temperature increase during sonication. Nearly 8 $\log_{10}$ reduction was observed for *E. coli* isolate from treated WWTP effluent using a 24 kHz ultrasound within 15 min at intensity of 250 W/L. Ultrasound disinfection had resulted in lower repair rates for all four *E. coli* isolates (Table 3.1 and 3.2). For ultrasound treated cells, the maximum percent log repair for *E. coli* isolate from WWTP after 24 h of repair was 53.81±0.23%.

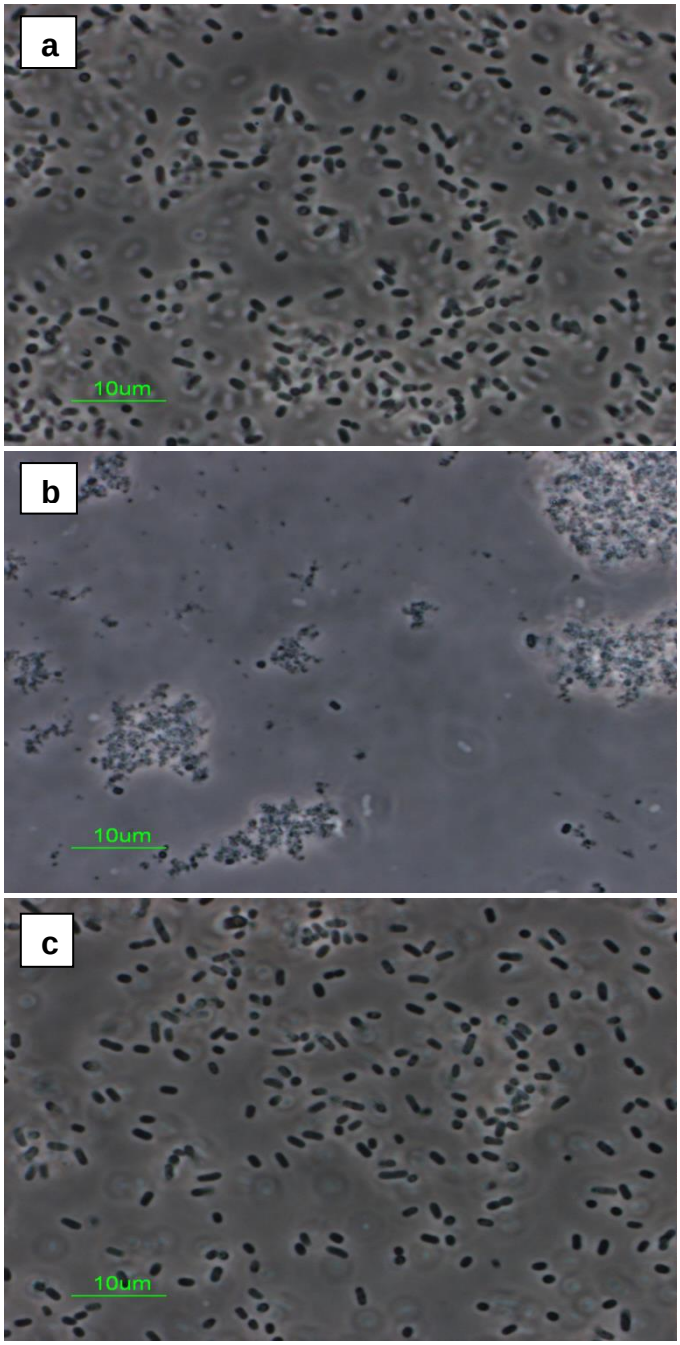


Figure 3.6 Transmission electron micrographs of *E. coli* (ATCC® 10798): (a) untreated, (b) ultrasounicated, and (c) UV-C treated *E. coli* cells. Transmission electron micrographs were obtained using a JEOL 1200ex transmission electron microscope by Microscopy Imaging Center at Texas A&M University.

Elevated temperature and mechanical shearing (Figure 3.6b) might be the cause for lowering the repair in *E. coli* cells even after 24 h of repair, in contrast to UV-C exposed cells, where no temperature increase and mechanical shear (Figure 3.6c) were observed. Similar findings were reported by Madge and Jenson (2002), where they had shown inhibition of cell's ability to repair damages after ultrasound. Madge and Jensen (2002) have observed nearly 4 $\log_{10}$ reduction of fecal coliforms using a 20 kHz ultrasound at intensity of 700 W/L for 6 min. They have accounted nearly 52% of disinfection towards elevated temperature. Increase in water temperature during ultrasound disinfection might result in weakening of intermolecular bonds and readily forming cavitation bubbles. Cells become more susceptible to heat because of decrease in fluidity of cell membrane due to shearing stress of acoustic cavitation (Madge and Jensen 2002).

While high inactivation rate and lower metabolic activity of *E. coli* isolates after ultrasonication operated at 250 W/L for 15 min were observed in our study, Paleologou *et al*. (2007) reported that 24 kHz ultrasound at 160 W was inefficient in inactivating *E. coli* than UV-A. Longer contact time of 120 min was required to achieve 92.3% inactivation in their study (Paleologou *et al*. 2007). A 99.99% inactivation was observed only after 120 min of ultrasound exposure in presence of 25 to 50 mg/L H_2O_2 (Paleologou *et al*. 2007). They concluded that UV-A had resulted in permanently damaged *E. coli* cells as no bacterial reactivation was observed after 24 h. A temporary cell inactivation was implied by the employed ultrasound disinfection (24 kHz at 150 W lead to 97.6% inactivation after 60 min for total coliforms (TCs)), which had resulted in

complete regeneration of bacteria after 24 h. Paleologou *et al*. (2007) have recommended to couple ultrasound with other disinfection treatments, such as UV-C and chlorination.

In this study, complete inactivation was observed after 15 min of exposure to 24 kHz continuous ultrasound at 100 W input power. Minimal repair of ultrasound exposed *E. coli* cells was observed over a 24 h period in comparison to UV-C exposed cells. On an average, the net log reduction for ultrasound treated *E. coli* strains was 5 $\log_{10}$ in comparison to 2 $\log_{10}$ for UV-C disinfection. The extent of dark repair was much lower than photoreactivation of ultrasonicated treated *E. coli* isolates (Figure 3.1).

Ultrasound had severely damaged the dark repair mechanism of *E. coli* strains. It should also be noted that the *E. coli* isolate from WWTP effluent had repaired the maximum under photoreactivating conditions after ultrasound and UV-C disinfection treatments. However, the percent log repair of *E. coli* isolates were nearly 53% for ultrasound disinfection and 83% for UV-C treatment after 24 h of disinfection. The magnitude of photoreactivation and dark repair of *E. coli* cells after UV-C disinfection was much higher than ultrasound treated cells (Figure 3.3 and 3.1). Photoreactivation seems to be a dominant repair mechanism for both ultrasound and UV-C treated *E. coli* cells. This observation is also supported by metabolic activity of the *E. coli* cells after ultrasound and UV-C disinfection. The metabolic activity of ultrasound exposed *E. coli* cells was lower than for UV-C exposed cells (Figure 3.5). Ultraviolet irradiated cells were able to maintain and/or recover their metabolic activity during dark and photoreactivation periods.

Ultrasound causes morphological damage to cells by shearing and breaking of cell membrane through mechanical forces arising from acoustic cavitation (Paleologou *et al*. 2007; Cameron *et al*. 2008). We have also observed similar morphological damages in *E. coli* cells treated with ultrasound. Shearing effects of ultrasound on *E. coli* cells can be clearly seen from transmission electron micrographs (Figure 3.6b). Reduction in cell size was evident for ultrasound exposed *E. coli* cells in comparison to UV-C treated cells which remained intact. Ultraviolet irradiation (UV-C) inhibits cell replication by altering the cellular DNA and does not have any morphological effect on cell wall, while ultrasound causes morphological damage to cell membrane which might have adversely affected the metabolic activity of the cells. Ultrasound might have caused internal cell damage which could have adversely affected repair mechanisms of *E. coli* cells.

CHAPTER IV

EFFECTIVENESS OF 24 kHz CONTINUOUS ULTRASOUND, LOW PRESSURE ULTRAVIOLET IRRADIATION, AND PHOTOCATALYSIS ON INACTIVATION OF *AEROMONAS HYDROPHILA* IN WATER

4.1 Overview

Aeromonas hydrophila is an emerging pathogen listed on the US Environmental Protection Agency's contaminant candidate list. Even at low concentrations found in drinking water, it has caused several gastroenteritis outbreaks. *Aeromonas hydrophila* has developed resistance to antibiotics which were used during the outbreaks. It has also been isolated from chlorinated drinking water. Alternative technologies are required for inactivation of *Aeromonas* spp. in drinking water. In this study, bactericidal effects of 24 kHz ultrasound, ultraviolet (UV-C) irradiation, and photocatalyst (TiO_2) were studied on inactivation of *Aeromonas hydrophila* (ATCC® 35654). Metabolic activity assay was performed to assess the activity of the microbial cells after disinfection treatments. The metabolic activity as measured using the AlamarBlue® assay was greatly lowered by the application of sonication and remained low even after 24 h of treatment. Ultrasound appeared to be an effective means of inactivating *Aeromonas hydrophila* by significantly decreasing the metabolic activity.

4.2 Introduction

Disinfection is the most important step in water treatment where microbial pathogens are inactivated to reduce the widespread of any waterborne diseases. *Aeromonas hydrophila* is an emerging pathogen listed on the US EPA's Candidate Contaminant List (CCL) (Chauret *et al*. 2001; Silvestry-Rodriguez *et al*. 2007; Chopra *et al*. 2009). *Aeromonas hydrophila*, a Gram negative bacterium is a fish pathogen and an opportunistic human pathogen (Fernández *et al*. 2000; Chauret *et al*. 2001; Chopra *et al*. 2009; Khan *et al*. 2012). For many years, *Aeromonas* spp. were considered opportunistic pathogens because they were associated with wound infections, septicemia, red fin diseases, and hemorrhagic septicemia peritonitis in immune-compromised patients (Sisti *et al*. 1998; Massa *et al*. 1999; Fernández *et al*. 2000; Chauret *et al*. 2001; Chopra *et al*. 2009; Khan *et al*. 2012). Several species of aeromonads have been indicated to act as enteropathogens, which are associated with production of toxins and adherence to epithelial cells (Sisti *et al*. 1998; Fernández *et al*. 2000; Lontsi *et al*. 2013).

Aeromonas hydrophila has caused several drinking water related gastroenteritis outbreaks when present in low concentrations from 1 to 10^4 CFU/100 mL (Fernández *et al*. 2000; Silvestry-Rodriguez *et al*. 2007; Lontsi *et al*. 2013). *Aeromonas* spp. has been found in domestic water supply even after chlorination and found to be potentially enteropathogenic (LeChevallier *et al*. 1982; Kühn *et al*. 1997; Sisti *et al*. 1998; Massa *et al*. 1999; Chauret *et al*. 2001; Chopra *et al*. 2009). Other disinfection treatments such as ozonation, ultraviolet, and ionizing radiations are also ineffective in decontaminating emerging chlorine resistant microorganisms (Furuta *et al*. 2004). Presence of *Aeromonas*

hydrophila in biofilms in drinking water supplies might pose potential gastroenteritis outbreaks (Fernández *et al*., 2000; Furuta *et al*. 2004; Lontsi *et al*. 2013). If contaminated water is ingested by a person, *Aeromonas hydrophila* might not be treated with antibiotics due to its resistance towards various antibiotics such as ampicillin, tetracycline, chloramphenicol, and sulphonamides (Chopra *et al*. 2009; Khan *et al*. 2012). It is critical to develop effective disinfection methods for *Aeromonas hydrophila*.

Ultrasound disinfection is an attractive means to enhance biological water quality as it is simple and causes no toxic by-products (Furuta *et al*. 2004). Use of ultrasound for wastewater disinfection, in combination with ultraviolet, hydrogen peroxide or ozone has been studied and demonstrated in many recent studies (Drakopoulou *et al*. 2009). Ultrasound waves are generated by mechanical vibrations of frequencies between 20 kHz and 800 kHz. Propagation of theses waves into liquid media results in production of alternating compressions and rarefactions. When the amplitude of the ultrasound wave is high, cavitation occurs. Cavitation is a phenomenon leading to creating and breaking of microbubbles. It is the collapse of these microbubbles which generates mechanical forces, thereby resulting in thinning and shearing of microbial cell wall (Joyce *et al*. 2003; Blume and Neis 2004; Furuta *et al*. 2004; Cameron *et al*. 2008; Drakopoulou *et al*. 2009). High power (10 W/cm^2 to 1000 W/cm^2) ultrasound at low frequencies (20 kHz to 100 kHz) is an effective means for disintegrating bacterial cells due to its ability to cause cavitation (Piyasena *et al*. 2003; Cameron *et al*. 2008). Ultrasonic application helps in decreasing the size of particle agglomeration as well (Joyce *et al*. 2003; Blume and Neis 2004; Furuta *et al*. 2004; Cameron *et al*. 2008).

In order to achieve a high log reduction or 100 % kill rates just by ultrasound, it is necessary to use high intensities, i.e. power to volume ratio (Joyce *et al*. 2003; Drakopoulou *et al*. 2009). Utilization of high power ultrasound on large scale disinfection is the limiting economic factor. A possible combination of a catalyst, such as titanium dioxide (TiO_2) with ultrasound might make the process more cost effective by lowering the exposure time (Drakopoulou *et al*. 2009). Ultrasound has also been considered as an alternative energy source for excitation of TiO_2 (Kubo *et al*. 2005; Drakopoulou *et al*. 2009). Kubo *et al*. (2005) evaluated ultrasound disinfection of *E. coli* in presence of TiO_2. Not much research has been done on application of ultrasound and ultraviolet (UV-C) along with photocatalysis on inactivation of *Aeromonas hydrophila*. In this study, bactericidal effects of 24 kHz ultrasound and ultraviolet (UV-C) irradiation in combination with titanium dioxide (photocatalyst) were studied to inactivate *Aeromonas hydrophila* (ATCC® 35654).

Disinfection treatment inactivates a microbial cell through multiple effects, such as DNA damage, thinning and disruption of cell membrane. Conventional culture based method has limitations in determining the cell viability. Hence, either cell viability assay or metabolic activity assay should be incorporated while assessing disinfection efficiency. AlamarBlue® is a vital dye which is non-toxic to cells and has been used for *in-vitro* viability determination. It has a higher sensitivity and reproducibility in detecting metabolic activities of cells even at low concentrations (O'Brien *et al*. 2000; Al-Nasiry *et al*. 2007). The active reagent of AlamarBlue®, resazurin, will be reduced to resorufin by healthy cells, which is fluorescent and pink in color. Till now, there has not

been enough research work done to assess metabolic activity of cells using AlamarBlue® dye after water disinfection. Evaluation of disinfection treatments through a culture based method and AlamarBlue® metabolic activity assay has been performed in this study.

4.3 Materials and Methods

4.3.1 Microorganism and media

Aeromonas hydrophila culture was obtained from American Type Culture Collection (ATCC® 35654). The culture was grown in Tryptic Soy Broth (TSB) overnight at 28±1°C. After 24 h, the culture was harvested by centrifugation at 10,000 × *g* for 3 min. The supernatant was removed and culture was re-suspended in 0.85% saline solution. The culture was washed twice to remove nutrients from the culture. The initial concentration of washed *Aeromonas hydrophila* was maintained at 10^8 cells/mL and was diluted 10 times in the batch reactor, described in the following sections. All the glassware, nuclease free deionzied (DI) water, and growth media were autoclaved prior to each experiment.

4.3.2 Titanium dioxide stock solution

Stock solution of titanium dioxide (10 g/L) was prepared by mixing 2 g of titanium dioxide powder (Degussa P25, Fisher Scientific, USA) in 200 mL nuclease free deionized (DI) water. The stock solution was not sterilized and stored in dark at room temperature. Before each experiment, the stock solution was homogenized for 3 min using the water bath sonicator.

4.3.3 Disinfection treatments

Ultrasonication

Ultrasound irradiation was provided by the horn type sonicator, operating at 24 kHz and 400 W (UP400S Hielscher, Germany). The experiment was conducted in 50 mL BD Falcon® centrifuge tubes. The working volume of the batch reactor was 40 mL, which included 0.85% saline solution and nuclease free DI water. The sonotrode was aligned to the center of the BD Falcon centrifuge tube. The distance between the tip of the sonotrode and bottom of the centrifuge tube was set at 1 cm. The temperature of the reactor was not controlled in order to study the combined effect of sonication and heat on inactivation of *Aeromonas hydrophila,* with final temperature reaching 65±5°C. The ultrasound power received by the *Aeromonas hydrophila* suspension in batch reactor at 100% amplitude and cycle one was 100 W, as estimated by calorimetric measurement. Calorimetric measurement was performed by recording water temperature at specific time intervals under ultrasound application in an insulated container. Energy required to heat up the water for a particular time interval was calculated using the specific heat capacity of water. The surface area of the horn type sonotrode H3 was 0.0706 cm^2 and the ultrasound intensity applied was 1416 W/cm^2 (ratio of power to sonotrode surface area).

The photocatalyst (TiO_2) was used in suspension form at three different concentrations: 0, 0.1, and 1 g/L. The batch reactors were subjected to ultrasound to achieve inactivation close to detection limit (10 CFU/mL). In order to accurately study the reactivation of microbial cells, complete inactivation was performed to eliminate

regrowth (for 100 μL plated on agar plates, at least one microbial colony should be observed). A control sample (with cells but not exposed to ultrasound) was used in the experiments. For negative control, nuclease free DI water with no cells was used. Horn type ultrasonicator was turned off after achieving complete inactivation of bacterial cells. The batch reactors were then exposed to visible light for photoreactivation to occur for 24 h. For dark repair, batch reactors were covered with aluminum foil for 24 h. All the ultrasonication experiments were performed once in triplicate at average pH 7.0±0.5 and 22±1°C. Regular time interval samples were taken during disinfection period at time t = 0, 5, 10, 15, and 20 min. Samples were taken after 24 h of photoreactivation and dark repair.

Ultraviolet irradiation

A UV-C low pressure (LP) germicidal lamp (Bryant energy, Indianapolis, USA) emitting peak wavelength of 254 nm was mounted horizontally in a polyvinyl chloride (PVC) box with dimensions of 81 × 61 × 51cm (L × B × H). The distance between UV lamp and water surface was kept at 12 cm. The average UV irradiance measured before sample exposure using a radiometer ranged from 3.25 to 3.27 mW/cm^2 (I_{avg}). The effect of water depth of 1 cm on absorbance of UV irradiance was neglected. The required UV dose was calculated using the following equation by adjusting the exposure time to achieve inactivation close to detection limit (10 CFU/mL) for all the treatments (Hallmich and Gehr 2010).

$$\text{UV dose (mW. s/cm}^2\text{)} = I_{avg}\ (\text{mW/cm}^2) \times \text{time (s)} \quad (4.1)$$

The experiment was conducted in 200 mL Pyrex® beakers. The working volume of 40 mL in batch reactor consisted of autoclaved DI water and 0.85% saline solution. Titanium dioxide was used in suspension form at three different concentrations: 0, 0.1, and 1 g/L TiO_2. The batch reactors were stirred continuously at 200 rpm using the magnetic stir plate and were irradiated under UV-C lamp to achieve inactivation close to detection limit. The whole setup was covered in a PVC box to cut off ambient light. A control sample without cells served as a negative control. Another control sample with cells (not exposed to irradiation) was also utilized during the experiments. After the required irradiation period, photoreactivation and dark repair were studied. The batch reactors were exposed to visible light for photoreactivation to occur for 24 h. For dark repair studies after disinfection, batch reactors were covered with aluminum foil for 24 h. Regular time interval samples were taken during disinfection period at time t = 0, 5, 10, 15, 20, 25, and 30 min. Samples were taken after 24 h of photoreactivation and dark repair periods. All the disinfection experiments were performed once in triplicate at average pH 7.0±0.5 and 22±1°C.

4.3.4 *Aeromonas hydrophila* cell enumeration and metabolic activity

Culture based method- plating

Aeromonas hydrophila concentrations were determined by the spread plate method. For this method, appropriate dilutions were performed in de-ionized and nuclease free water. At regular time interval sampling, 100 µL of the diluted sample was spread on the Tryptic Soy agar plates from each reactor. Triplicate plates were inoculated for each batch reactors. The plates were then incubated for 24 h at 28±1°C.

After incubation, average CFU/mL for all the triplicate experiments was recorded and used in the analyses.

Cell viability assay

Cell viability and metabolic activity of *Aeromonas hydrophila* were assessed by the AlamarBlue® cell viability assay. A 10X, AlamarBlue® cell viability reagent was used to assess the cell viability. Only 10% of the AlamarBlue® reagent, i.e., 10 µL was added to 100 µL of bacterial cells according to the manufacturer's protocol (Invitrogen, USA). The reagent was used in a 96 well plate format in a "no wash" mode. The 96 well plate was then incubated at 37°C for desired time to allow reduction of resazurin to resorufin. Samples with high cell counts were incubated for 2 h, whereas samples with low cell counts after disinfection were incubated for 24 h, in order to achieve reduction of resazurin to resorufin. AlamarBlue® added to medium without cells served as a negative control. After the desired incubation, the resulting fluorescence was read using a Victor 2 multi-label counter 1420 microplate reader at 560 nm (Wallac 1420 Victor 2, Perkin Elmer, Massachusetts, USA). The resulting fluorescence value from negative control was subtracted from the fluorescence reading for each sample.

4.3.5 Data analysis

The data were analyzed using statistical software, Design Expert®. Full factorial ANOVA model was selected in Design Expert® to analyze survival of *Aeromonas hydrophila* from culture based method for each disinfection treatment. This model consisted of two factors, factor A and factor B. Factor A had three levels of TiO_2 concentration, whereas factor B had five and seven levels for disinfection time under

ultrasonication and UV irradiation, respectively. A total number of nine replicates were chosen for the statistical analysis for each treatment. Type I error value of 0.05 was selected for conducting ANOVA.

4.4 Results

4.4.1 Survival of *Aeromonas hydrophila* during disinfection

Inactivation of *Aeromonas hydrophila* over time during ultrasound disinfection treatment is resented in figure 4.1. Only 5 min of ultrasound exposure with 1 g/L and without TiO_2 resulted in nearly 8 $\log_{10}$ reductions of *Aeromonas hydrophila* cells. Similar inactivation was observed in ultrasound with 0.1 g/L TiO_2 only after 15 min. *Aeromonas hydrophila* inactivation during ultraviolet (UV-C) disinfection is presented in figure 4.2. Maximum log reduction (7.61 $\log_{10}$) was achieved after 15 min of exposure under UV-C. Higher contact time was observed for treatments with TiO_2. Presence of photocatalyst did not increase the inactivation rate of *Aeromonas hydrophila*. Ultraviolet (UV) irradiation alone was effective in inactivating the cells with a UV dose of 2934 mJ/cm^2 for complete inactivation of *Aeromonas hydrophila* in absence of TiO_2. However, in presence of 0.1 g/L and 1 g/L TiO_2, UV doses of 3912 mJ/cm^2 and 5868 mJ/cm^2 were respectively applied to achieve complete inactivation close to detection limit (10 CFU/mL).

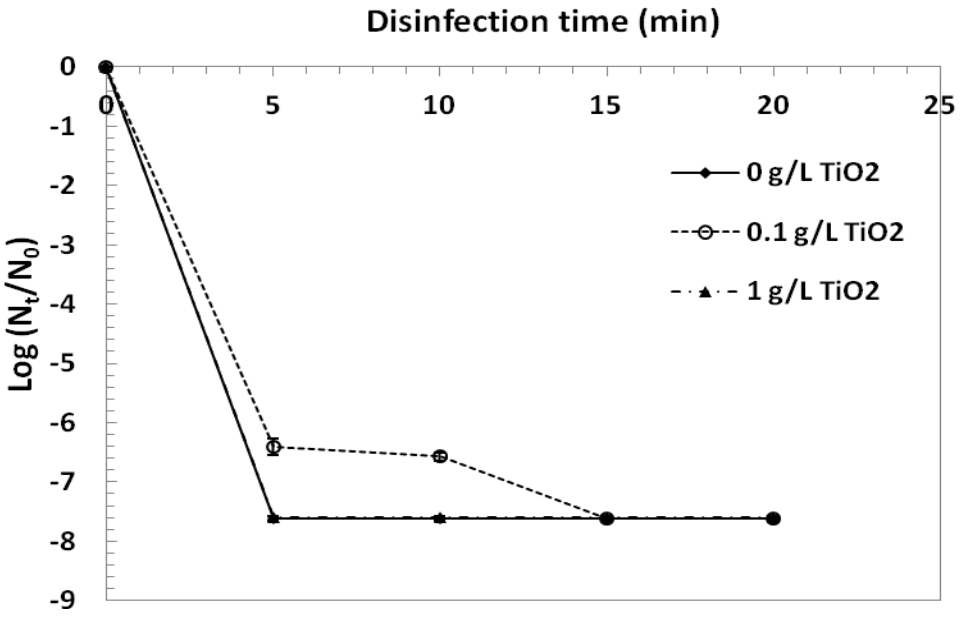


Figure 4.1 Survival curves of *Aeromonas hydrophila* (ATCC® 35654) during ultrasound and photocatalytic disinfection treatments with a detection limit of 10 CFU/mL. N_t and N_0 are bacterial cell concentrations at time t and 0 min, respectively. Standard deviation of three replicates is presented as error bar.

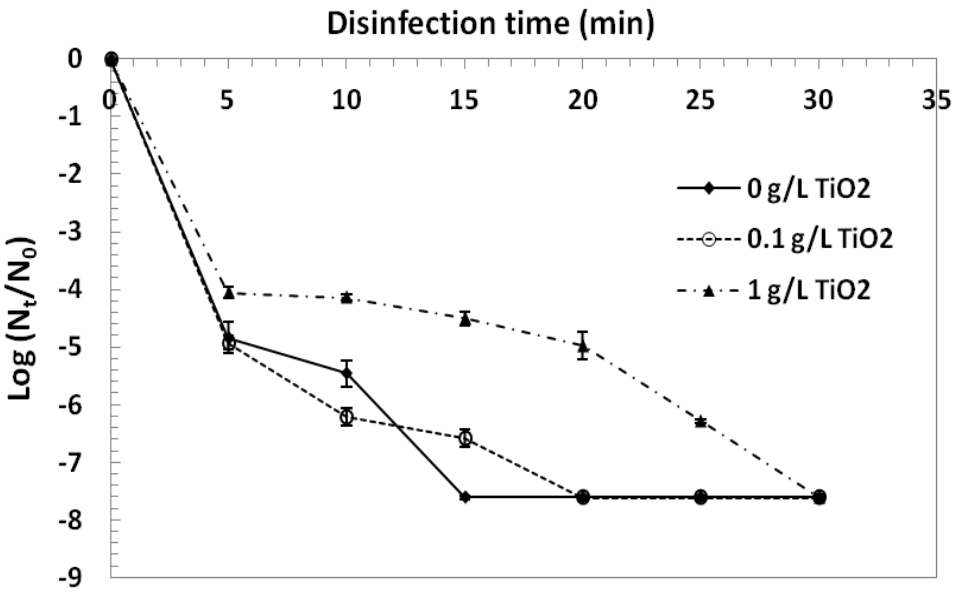


Figure 4.2 Survival curves of *Aeromonas hydrophila* (ATCC® 35654) during ultraviolet and photocatalytic disinfection treatments with a detection limit of 10 CFU/mL. N_t and N_0 are bacterial cell concentrations at time t and 0 min, respectively. Standard deviation of three replicates is presented as error bar.

In our study, we did not find significant difference in *Aeromonas hydrophila* log reduction in presence of TiO_2 for both ultrasound and UV disinfection treatments ($p > 0.05$, data not shown). There was approximately 1 $\log_{10}$ increase in *Aeromonas hydrophila* concentration after 24 h of ultrasound disinfection, with or without TiO_2. While there was 5 $\log_{10}$ increase in *A. hydrophila* cells after 24 h of UV-C disinfection, with or without TiO_2. Overall, dark repair of *A. hydrophila* was comparable to photoreactivation after corresponding disinfection treatments (Table 4.1).

Table 4.1 Log repair of *Aeromonas hydrophila* after 24 h of disinfection treatment

TiO_2 Concentration (g/L)	**Ultrasound disinfection**		**Ultraviolet disinfection**	
	Log dark repair	**Log photoreactivation**	**Log dark repair**	**Log photoreactivation**
0	1.32 ± 0.06[§]	1.47 ± 1.15[§]	5.31± 0.02[§]	5.30 ± 0.02[§]
0.1	1.10 ± 0.15[§]	1.03 ± 0.10[§]	5.38 ± 0.03[§]	5.42 ± 0.02[§]
1	1.13 ± 0.16[§]	1.40 ± 0.09[§]	5.35 ± 0.03[§]	5.39 ± 0.04[§]

[§]Standard deviation of three replicates is presented.

4.4.2 Metabolic activity of *Aeromonas hydrophila* during disinfection

The metabolic activity of *Aeromonas hydrophila* during ultrasound disinfection is presented in figure 4.3. Before exposure to disinfection, cells maintained an active metabolic activity. This could be observed from higher fluorescence values seen at 0 min. During ultrasound disinfection *Aeromonas hydrophila* cells were becoming metabolically inactive (Figure 4.3). Metabolic activity of *Aeromonas hydrophila* remained the same (close to control) even after 24 h of disinfection. The metabolic activity was greatly lowered by the application of ultrasound.

Ultraviolet irradiation had less pronounced effect on the metabolic activity of the *Aeromonas hydrophila* cells. Cells had maintained their activity during the UV-C disinfection period (Figure 4.4). Metabolic activity under dark and light were comparable after 24 h of UV disinfection treatment. The repair after 24 h was higher for *Aeromonas hydrophila* cells exposed to UV and UV induced photocatalysis (Figure 4.5).

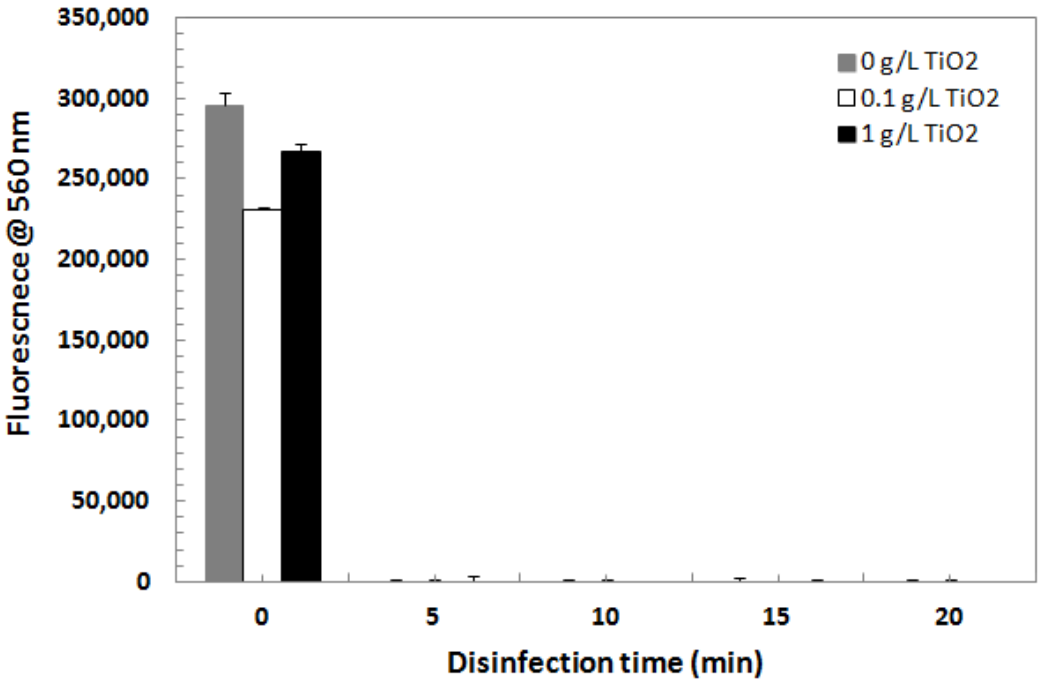


Figure 4.3 Metabolic activity of *Aeromonas hydrophila* as measured by AlamarBlue® fluorescence during ultrasonication and photocatalytic disinfection treatments. Values were deducted from the fluorescence of AlamarBlue® without cells. Standard deviation of three replicates is presented as error bar.

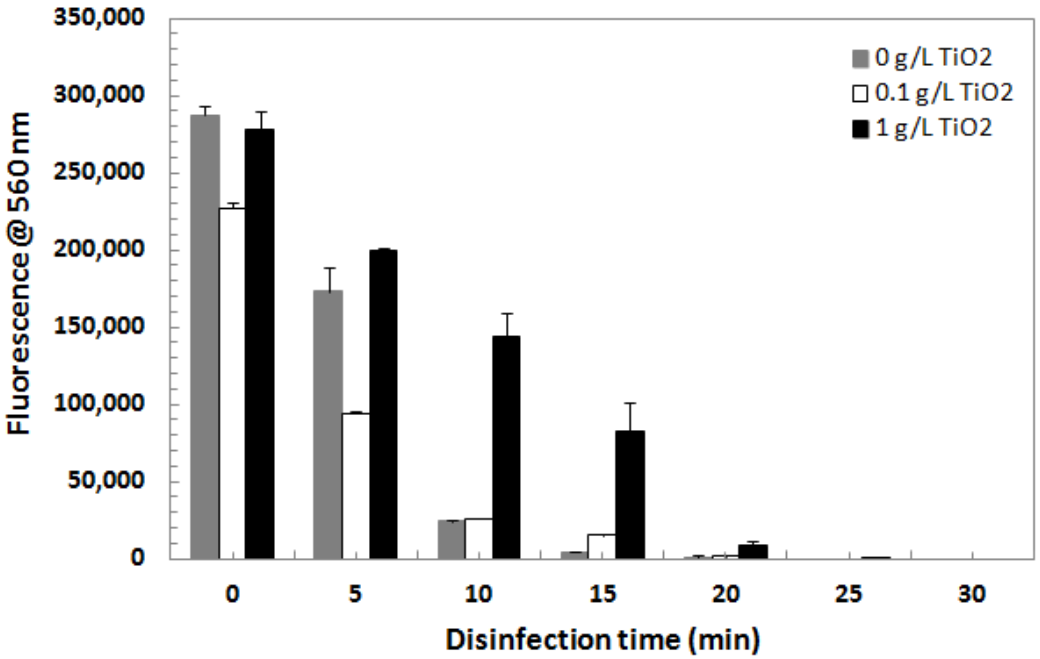


Figure 4.4 Metabolic activity of *Aeromonas hydrophila* as measured by AlamarBlue® fluorescence during ultraviolet and photocatalytic disinfection treatments. Values were deducted from the fluorescence of AlamarBlue® without cells. Standard deviation of three replicates is presented as error bar.

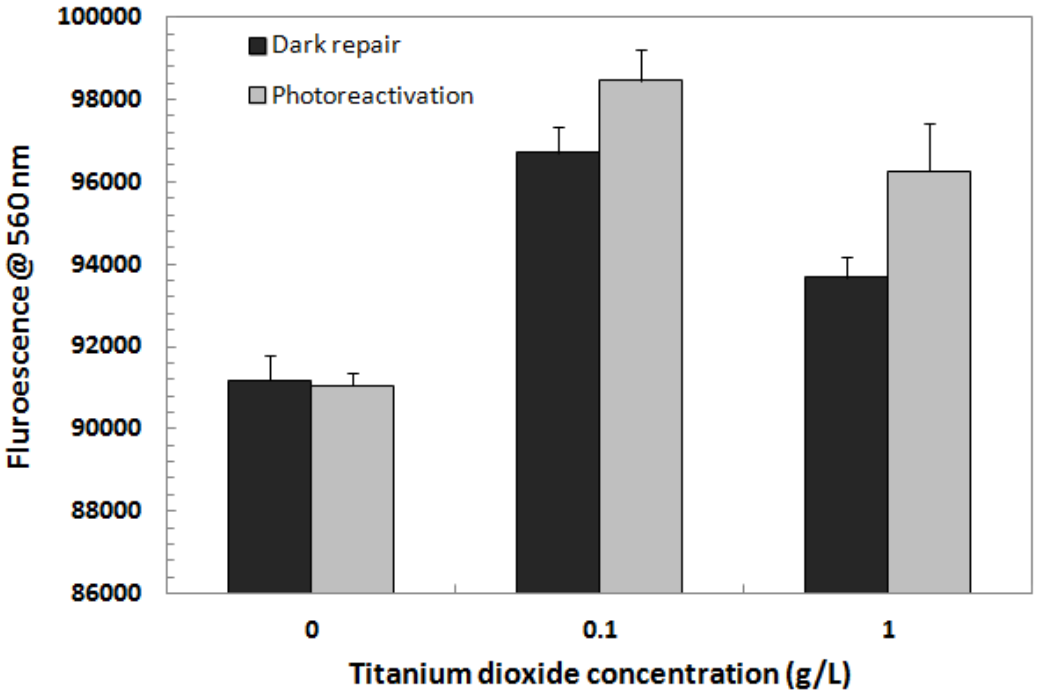


Figure 4.5 Metabolic activity of *Aeromonas hydrophila* after 24 h of disinfection treatment in terms of AlamarBlue® fluorescence for ultraviolet treated cells. Standard deviation of three replicates is presented as error bar.

4.5 Discussion

Earlier *Aeromonas* spp. inactivation studies focused mainly on chlorination (LeChevallier *et al*. 1982; Kühn *et al*. 1997; Sisti *et al*. 1998; Massa *et al*. 1999; Fernández *et al*. 2000; Chauret *et al*. 2001). Bactericidal effects of chlorine on *Aeromonas hydrophila* was evaluated by Sisti *et al*. (1998). They have reported resistance of *Aeromonas hydrophila* to the oxidizing activity of free chlorine to increase with water temperatures above 20ºC. Silvestry-Rodriguez *et al*. (2007) had studied the effect of silver on inactivation of *Aeromonas hydrophila* in tap water. Maximum log reduction (> 6-$\log_{10}$) was achieved only after 9 h of treatment. In the present study, two

possible combinations of physical (ultrasound and UV-C) and chemical (photocatalyst) disinfection methods were explored. Complete inactivation (7.61 $\log_{10}$ reduction) was achieved under UV-C irradiation at 32.6 W/m^2. The inactivation rate of 0.50 log min^{-1} was observed during UV-C irradiation. If photoreactivation and dark repair were taken into consideration, then nearly 2.3 $\log_{10}$ net reductions were observed after 24 h of repair period. *Aeromonas hydrophila* had repaired nearly 5.3 $\log_{10}$ during the 24 h period after UV-C treatment.

Aeromonas hydrophila inactivation was delayed in presence of TiO_2 than in absence under UV-C disinfection (Figure 4.2). The metabolic activity of *Aeromonas hydrophila* cells exposed to UV and UV induced photocatalysis was higher than ultrasound-treated cells. Titanium dioxide (TiO_2) at 1 g/L was not effective in lowering the metabolic activity of *A. hydrophila* cells; and this might be due to scattering of UV light and possible shielding of cells by TiO_2 particles. Suspended solids present in water could shield the bacteria from the effect of disinfection treatments, thereby decreasing the efficiency (Blume and Neis 2004).

Khan *et al*. (2012) had explored a possible combination of a physical and chemical disinfection, where a thin film fixed bead reactor utilizing combination of solar energy and TiO_2 was effective in inactivating *Aeromonas hydrophila* (ATCC® 35654). They showed that high sunlight intensities led to a greater proportion of inactivation in comparison to the low intensities (< 600 W/m^2). However, in our study we have observed high inactivation rate under UV-C in absence of TiO_2. This might be due to

utilization of a low pressure UV, instead of a wide range of wavelength source, such as medium pressure UV or sunlight and TiO_2 in suspension.

A complete inactivation of nearly 8 $\log_{10}$ reduction was observed within 5 min of exposure at an inactivation rate of 1.52 log min^{-1} for ultrasound at 0g/L and 1 g/L TiO_2. *Aeromonas hydrophila* (ATCC® 35654) was inactivated in water using 24 kHz ultrasound using a horn type sonotrode H3 of 0.0706 cm^2 at 1416 W/cm^2 and 250 W/L. Application of ultrasound had greatly lowered the repair rate of *Aeromonas hydrophila* and resulted in nearly 1 $\log_{10}$ increment in cell counts after 24 h of disinfection. The metabolic activity of the cells was at a low level as AlamarBlue® fluorescence was close to the negative control fluorescence. This indicates that cells were no longer metabolically active and were damaged by the synergistic effect of shock waves and localized heating. This observation is in agreement with the data obtained by culture based method (Figure 4.1). It was expected that presence of photocatalyst would enhance the inactivation of *Aeromonas hydrophila* due to production of hydroxyl radicals and oxidation of cell membrane. Generation of free radicals, such as hydroxyl radicals occurs in presence of TiO_2 particles and ultrasound energy (Paleologou *et al*. 2007). Presence of titanium dioxide with ultrasound disinfection did not have significant effect on *Aeromonas hydrophila* inactivation. This observation is contrary to a previous study by Kubo *et al*. (2005), where higher inactivation rate of *E. coli* cells in presence of 5 g/L TiO_2 than in absence, at 500 W/L and 50°C was observed. Bactericidal effect of ultrasound is categorized by mechanical effects caused by cavitation and chemical effects of cavitation due to generation of free radicals (Hulsmans *et al*. 2010). Exact

mechanisms of microbial inactivation due to cavitation have not been convincingly established. However, mechanical effects are more pronounced and are observed for microbial inactivation. Membrane damage due to shear is a possible mechanism for cell death during ultrasound cavitation (Joyce *et al*. 2003; Blume and Neis 2004; Furuta *et al*. 2004; Cameron *et al*. 2008; Broekman *et al*. 2010).

In this study, a slight increase in *A. hydrophila* cell counts was observed after ultrasound disinfection. There may be other repair mechanisms happening inside the cell which repair the membrane damage due to mechanical shear and temperature stress. However, the repair was only 1 $\log_{10}$ even after 24 h. Other reason for this 1 $\log_{10}$ repair could be due to the presence of unsterilized titanium dioxide suspension while conducting photocatalytic disinfection experiments.

In general, physical disruption of microbial cell membrane is considered as the main mode of inactivation of microbes (Furuta *et al*. 2004). The underlying principle of sonication lies in acoustic cavitation, in which microbubbles are formed and collapsed in milliseconds. Thus extreme temperature and pressure gradients are created which result in mechanical shear (Piyasena *et al*. 2003; Drakopoulou *et al*. 2009). Ultrasound can serve as an attractive means to inactivate *A. hydrophila* cells with lower repair rates after disinfection.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Reactivation of microorganisms after disinfection treatments poses a major risk for drinking water safety and associated health effects. Efficiency of a disinfection system is adversely affected by the reactivation of microorganisms. Microorganisms often tend to lose their culturability after disinfection, but could remain viable and proliferate under optimum conditions. Presence of viable but non-culturable (VBNC) state cells in disinfected water could pose a potential health risk. Accurate estimation of VBNC cells through molecular methods is critical.

In this work, inactivation and reactivation of laboratory *E. coli* strain, environmental *E. coli* isolates, and an emerging waterborne bacterium (*Aeromonas hydrophila*) have been studied. Dark repair and photoreactivation of bacterial cells were assessed after photocatalysis, ultraviolet irradiation (UV-C), and ultrasound disinfection treatments. Culture based methods in combination with molecular assays were utilized to assess the bacterial inactivation and potential repair.

Major conclusions from this research work are:

(1) Flow cytometry had provided accurate state of microorganisms by enabling fast and reliable counts of live and dead percentages of *E. coli* present in water after disinfection. Titanium dioxide had slightly lowered the percent log repair and repair rate

(log h^{-1}) of *E. coli* strain (ATCC® 10798) during photoreactivation and dark repair after UV-C assisted photocatalytic disinfection.

(2) Photocatalytic disinfection with 0.1 g/L of TiO_2 had resulted in better disinfection (log reduction) and lower *E. coli* (ATCC® 10798) repair rate in comparison to UV-C disinfection. However, increasing photocatalyst concentration did not significantly reduce the photoreactivation and dark repair of *E. coli* over a 4 h period.

(3) Ultrasound reduced the reactivation of *E. coli* isolates in comparison to UV-C disinfection. Transmission electron micrographs of ultrasound treated *E. coli* cells showed morphological damage to cell membrane. Lethal damage to *E. coli* cells by shearing and breaking of cell membrane might have adversely affected cell's repair mechanisms.

(4) Metabolic activity of *E. coli* strains was greatly reduced due to morphological damage to cell membrane caused by 24 kHz continuous ultrasound in comparison to UV-C.

(5) Ultrasound significantly decreased *Aeromonas hydrophila* reactivation in comparison to UV and UV induced photocatalysis.

(6) Addition of TiO_2 to ultrasound and UV-C disinfection did not enhance *Aeromonas hydrophila* inactivation rate (log min^{-1}). Ultrasound irradiation had resulted in faster inactivation rate of 1.52 log min^{-1} and complete inactivation within 5 min. Only 1.4 $\log_{10}$ repair was observed for ultrasound treated cells in contrast to 5.3 $\log_{10}$ repair for UV-C treated cells.

Based on experimental data and TEM micrographs, it can be concluded that ultrasound is a promising disinfection alternative to inactivate environmental *E. coli* isolates and *Aeromonas hydrophila* in water. Ultrasound decreased the metabolic activity of *Escherichia coli* and *Aeromonas hydrophila* cells as well as photoreactivation and dark repair mechanisms.

5.2 Future Recommendations

In this research work, reactivation mechanisms of *Escherichia coli* and *Aeromonas hydrophila* were studied using flow cytometry and AlamarBlue® assay. Further molecular investigations of disinfection effects of ultrasound and reactivation of *E. coli* and *A. hydrophila* should be conducted. Reactivation studies using pulse-ultrasound can be assessed for its effectiveness in decreasing bacterial reactivation. Various combination of ultrasound with immobilized photocatalyst, UV-C, and medium pressure UV can be explored in future studies. Continuous flow type reactors for UV-C and ultrasound disinfection combination treatment can be evaluated. Ultrasound is an energy intensive process and possible combination with low pressure UV-C may decrease the energy cost, making it more economical on a large scale. Pilot scale studies are required to determine economic feasibility of the above stated combinations.

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