INVESTIGATION ON METHODS AND MECHANISMS OF BACTERIAL REDUCTION IN AGRICULTURAL WASTEWATER

A Dissertation

by

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ABSTRACT

Large animal farms produce tremendous amounts of manure and manure are obvious sources of pathogenic bacteria. In addition, antibiotic consumption in modern livestock industry has significantly increased, resulting in a substantial presence of residual antibiotics in the manure and manure effluent. As a doctoral research dissertation, two methods using 1) the activated iron media (AIM) created from zero-valent iron (ZVI) and aqueous ferrous ion (Fe^{2+}); and 2) cationic polymers, were investigated in terms of their ability to remove those contaminants in water system. The research is composed of two chapter: 1) effect of AIM on indicator bacteria removal and its bacterial removal mechanism; 2) effects of cationic polymers on manure separation, bacteria and antibiotic-resistant bacteria, and its synergistic effect against *E. coli* removal when the polymer is applied with hydrogel. The results obtained from this study will help us with better understandings of the fate of bacteria and antibiotics in animal farm industry.

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NOMENCLATURE

AIM	Active Iron Media
ANOVA	Analysis of Variance
BA	Sodium Benzoate
CFU	Colony forming unit
COD	Chemical Oxygen Demand
CTC	Tetrazolium salt 5-cyano-2,3-ditolyltetrazolium chloride
DAPI	4’,6-diamidino-2-phenylindole
DDI	Deoxygenated and deionized
DI	Deionized
DMSO	Dimethyl Sulfoxide
DO	Dissolved Oxygen
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetate
Fe^T	Total Fe
FFA	Furfuryl Alcohol
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
IEP	Isoelectric Point
LB	Luria-Bertani
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration

MnTBAP	Manganese (III) tetrakis (4-benzoic acid) porphyrin
MPN	Most Probable Number
MS	Mass Spectrometry
NP	nano Particle
n-ZVI	nano Particulate Zero Valent Iron
OD	Optical Density
PAM	Polyacrylamide
p-HBA	4-hydroxybenzoic acid
Poly-gel	Hydrogel Containing Polymer
ROS	Reactive Oxygen Species
RPM	Revolutions per minutes
SA	Sodium Azide
SAS	Sodium Azide Spiked
SP	Sodium Pyruvate
TEM	Transmission electron microscopic
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UV	Ultraviolet
XTT	2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
ZVI	Zero Valent Iron

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CHAPTER I

INTRODUCTION

I.1. Background: Microbial Contamination from Animal Feeding Operations and Implications for Water Quality

American agriculture has significantly changed its structure since 1950. Across all farm operations and sectors have expanded, which means size of farms is getting larger but fewer in their number. This shift is most noticeable in the production of livestock (USEPA, 2013). For example, fully grown lactating cow produces approximately 68 L of manure daily (Lorimor et al., 2004). Based on this estimation, large dairy farms with 4,000 cows generates about 100,000 m^3 of manure each year (Liu et al., 2015). As a result, Over the past 70 years, the livestock production in the United States has more than doubled; however, the number of operations has decreased by 80% (Graham and Nachman, 2010). The reason of this increase is to meet the demands for meat and animal products from a growing human population in the U.S. and abroad. Historically, animal manure has been used as a great nutrient source and soil conditioner for crop production. Therefore, in the agricultural field, land application of manure effluent and solids is a common practice in crop production because livestock manure is an important agricultural resource that contains a high level of essential nutrients and organic matter (Islam et al., 2004; Ribaudo et al., 2003). However, as production has shifted to much larger farms, concentration of livestock has also increased, resulting in the need of more intensive management practice to treat increased concentration of animal manure because the excess land application of manure may exceed the needs of the plants and the crops. U.S. Department of Agriculture's Economic

Research Service (USDA ERS) reported that greater than 70% of phosphorus and 60% of nitrogen in the manure cannot be assimilated in the crop land. In particular, runoff related to manure is considered a major contributor to widespread nutrient contaminations, resulting in water quality pollutions.

While manure's contributions to nutrient water quality impairment is a commonly recognized issue, contaminations caused by manure and livestock application also need to be considered. Manure often contains human and animal infectious pathogens, heavy metals, antimicrobials, and hormones that can be introduced to surface and ground water through runoff and permeation which can impact aquatic ecosystem and water conditions (Gullick et al., 2007; Rogers, 2011). In particular, applying manure with high levels of pathogens could pose health risks to animals and humans. There are numerous bacteria species present in manure, among which the most common pathogenic microorganisms found in manure include *Salmonella*, toxigenic *Escherichia coli* (*E. coli*), *Yersinia* and *Campylobacter* species, protozoa *Giardia,* and *Cryptosporidium* (Bicudo et al., 2003). Because disease outbreak caused by pathogens can pose a serious threat to public health, there have been many studies and researches on methods to reduce those pathogen levels in manure such as chemical treatment using synthetic polymers (Liu et al., 2016) or biological treatment such as activated sludge process (Vanotti et al., 2005).

I.2 Active Iron Media

Applying zero-valent iron (ZVI) to treat contaminated soils and groundwater have been becoming popular in the past two decades. Substantial experiences have been accumulated on kinetics of iron corrosion product/oxides and contaminant destruction. In addition, there also have been extensive researches on effect of ZVI performance, monitoring, modeling, and design (Blowes et al., 2000; Huang et al., 2003; Morrison et al., 2002; Shokes and Moller, 2000).

The known mechanisms of removing contaminants by ZVI in aquatic system were reduction, precipitation, and adsorption. Despite these well-known capabilities of ZVI and the underlying chemistries, applying ZVI for environmental remediation in the real field is much more difficult, mainly because surface passivation problems of ZVI, i.e., rapid loss of ZVI reactivity due to the tendency of forming passive iron oxide coatings on the ZVI grain surface once ZVI comes into contact with water and/or soils. Fe^0, a relatively strong reductant, can be readily reduced not only by the reactive target contaminants (such as nitrate, TNT), but also by O_2, or even H_2O (e.g., $Fe^0 + 2\ H_2O \rightarrow Fe^2 + 2\ OH^- + H_2$). These redox reactions, accompanied by iron corrosion, will result in the formation of an iron rust coating that is often chemically passive to varying degrees and subsequently diminishes the overall reactivities of ZVI. There have been extensive efforts to overcome the passivation problem. For example, addition of sand with ZVI can prevent iron passivation because contaminants compete against Fe (II) and Fe (III) generated from ZVI when the contaminants adsorbed onto sand particles; addition of MnO_2 can prevent passivation through redox reaction (Fe (II) generated from ZVI oxidation are used for reducing MnO_2 instead of producing iron corrosion products); activated carbon keeps ZVI reactivity as it is able to transfer electrons from iron corrosion products to contaminants (Btatkeu-K et al., 2014; Dos Santos Coelho et al., 2008; Luo et al., 2014; Noubactep et al., 2011; Scherer et al., 2000; Shi et al., 2014).

In this dissertation, a specific technique to prevent iron passivation problem was used by pre-treatment (preconditioning) of the ZVI with nitrate and ferrous iron as it was developed in the previous study (Huang and Zhang, 2005). The previous study demonstrated that magnetite was the dominant corrosion product as a result of iron-nitrate redox reaction (preconditioning). The reaction during the ZVI preconditioning was described as (Huang and Zhang, 2005);

$$NO_3^- + 2.82\ Fe^0 + 0.75\ Fe^{2+} + 2.25\ H_2O \rightarrow NH_4^+ + 1.19\ Fe_3O_4 + 0.50\ OH^- \quad (1)$$

The strategy of augmenting the ZVI system with externally added Fe^{2+} to overcome ZVI surface passivation was highly effective (Huang et al., 2012; 2013). The preconditioning process is to create a mixture of highly reactive iron-based media consisted of ZVI grains coated with a reactive iron oxide coating and a substantial amount of discrete and highly reactive iron oxide phase that features a magnetite-like structure with non-stoichiometric and flexible Fe(II)-Fe(III) compositions. The preconditioning process can overcome surface passivation of ZVI, thus enhance the reactivity of the ZVI and increase removal efficiency of contaminants. The previous study concluded that both Fe^{2+} and Fe_3O_4 (magnetite) generated from preconditioning process were important for pollutants degradation by ZVI (Huang et al., 2012; 2013; Tang et al., 2016). The effects of pre-treated ZVI (hybridized ZVI/Fe_3O_4/Fe^{2+}, also known as the Activated Iron Media) on removal of various contaminant in water systems have been demonstrated. However, very importantly, its effect against bacteria levels in wastewater has not been evaluated yet. Although some studies have reported study results of bactericidal effect of zero valent iron, most of the studies have focused on the use of nano-scale ZVI particles, not micron-scale ZVI media that are used to create the activated iron media (Diao and Yao, 2009; Kim et al., 2010a; Lee et al., 2008a; Noubactep, 2011; Xu et al., 2013). The effect of the activated iron media on bacterial removal has not been assessed yet.

I.3 Coagulation and Flocculation

Some suspended particles cannot be removed completely by plain gravitational settling. Large, heavy particles settle out readily, but smaller and lighter particles settle very slowly or in some cases do not settle at all. Because of this, the sedimentation step is usually preceded by

adding chemical (coagulant)known as coagulation. Coagulants added to the water to bring the non-settling particles together into larger, heavier masses of solids called floc. Coagulation is usually accomplished in two stages, rapid mixing and slow mixing. Rapid mixing helps to disperse the coagulants so that the chemical can be homogenized in the water, and to ensure a complete chemical reaction. After the rapid mix, a longer period of gentle agitation is required to increase particle collisions and enhance the size of flocs. This slow mixing procedure to make a floc is called flocculation.

Coagulation and flocculation occurs in consecutive steps, allowing particle collision and growth of floc. This is then followed by sedimentation. If coagulation is incomplete, flocculation step will be unsuccessful, and if flocculation is incomplete, sedimentation will be unsuccessful. Coagulant chemicals have charges opposite those of the suspended solids in the water to neutralize the negative charges on the solids surface. Once the surface charge of the particles is neutralized, the small-suspended particles can aggregate together.

Flocculation, a gentle mixing stage, increases the particle size from submicroscopic microfloc to visible suspended particles. Microfloc particles collide, causing them to bond to produce larger, visible flocs called pinflocs. The pinflocs continues to build with additional collisions and interaction with added chemicals such as coagulant. Macroflocs are formed and high molecular weight polymers, called coagulant aids, may be added to help bridge, bind, and strengthen the floc, add weight, and increase settling rate. Once floc has reached it optimum size and strength, water is ready for sedimentation.

I.4 Cationic Polymer

A cationic polymer is a long chain molecule with repeating units such as acrylamide, which have a net external positive charge. As relatively high molecular weight polyelectrolytes,

polymers aid flocculation. It is believed that polymer molecules attach (or adsorb) to the surface of two or more particles by some specific chemical interaction and form bridges between them. Large polymers are needed to accomplish destabilization by this mechanism, because the polymer bridge has to span two diffuse layers (Benjamin and Lawler, 2013).

Besides the function of polymer as coagulant, cationic polymers are promising antimicrobial agents (Carmona-Ribeiro and de Melo Carrasco, 2013). Positive charges of the polymer have been used as antimicrobial agents by themselves and/or in sophisticated formulations (Tapias et al., 1994). The following sequence of events occurs with microorganisms exposed to cationic agents: (i) adsorption and penetration of the agent into the cell wall; (ii) reaction with the cytoplasmic membrane (lipid or protein) followed by membrane disorganization; (iii) leakage of intracellular low-molecular-weight material; (iv) degradation of proteins and nucleic acids; and (v) wall lysis caused by autolytic enzymes (Salton, 1968; Denyer, 1995).

Larger farms are increasingly processing manure before land application in order to lower hauling costs, decrease environmental pollution risk from nutrient losses to water, and comply with federal and state regulations. Therefore, a comprehensive manure handling and treatment strategy is crucial on a large dairy farm. A typical manure management strategy is liquid/solid separations through a variety of operations such as screens, presses, or centrifuges to produce solids with rich nutrients and a liquid stream with low-nutrients/solids. The properly separated liquid can be used for irrigation or reused as process water to flush the barns. Closing the waste cycle on dairy farms worldwide can increase the sustainability of such operations; however, research into factors that could lead to “unforeseen” consequences from changes in management practices is essential to ensure the efficacy of such changes.

On large farms, physical separation methods such as centrifugation are often enhanced by chemical addition (Vanotti et al., 2002; Amuda and Alade, 2006; Liu et al., 2017) because physical separation alone is not suitable to remove fine suspended particles, which typically contain the majority of the nutrients, from recycled liquid streams (Liu et al., 2016). These chemicals bind and separate the smaller particles for efficient concentration of solids and nutrients (Zhang and Westerman, 1997). For example, the use of polyacrylamide (PAM) polymers, their homo-polymers, and their acrylamide/acrylic acid co-polymers, alone or in combination with various inorganic salts, have proven to be effective in enhancing concentration of solids and nutrients in the separation process (Vanotti et al., 2002). Despite the efforts of recent studies examining manure separation, there is still a lack of studies relating polymer effect on pathogen indicator reduction, as well as dairy manure characteristics such as the level of total solids, chemical oxygen demand (COD), and liquid-solid separation efficiency of raw manure. Furthermore, there is little information of the effects of polymers on bacterial concentration and especially on antibiotic resistant bacteria in a raw manure and in the liquid stream of polymer treated manure.

I.5 Antibiotics in Manure

In addition to the microbial contamination, plenty of many different pharmacologically active substances (e.g., antibiotics) have been being used annually to prevent and treat animal diseases. Antibiotics used in farm operations are specifically designed to control bacteria in animal production and treat diseases caused from bacterial infection (Timothy et al., 2012). For example, foot rot (or hoof rot) is a common microbial infection in livestock that causes swelling, fever, and inflammation on the hooves, resulting in severe lameness (AABP, 2016). Therefore, antibiotics have been essential in treating diseases caused by bacterial infection (Kumar et al.,

2005). However, it has now become clear that the extensive use of antibiotics can cause various problems in both environmental and public health perspectives. The major concern is that excess use of antibiotics could lead to the appearance of new strains or mutated bacteria that are resistant to these antibiotics, thus threatening human health (Solomons, 1978).

In terms of livestock operation, although most antibiotics are used for the treatment of infections, a significant portion of these are also used in animal feed as a supplement to promote growth. The use of antibiotics for animal growth promotion is not new; these pharmaceuticals were approved in the United States and United Kingdom in 1949 and 1953, respectively (Witte, 2000). Antibiotics in animal feed helps increase the animal's ability to absorb feed and thus reach market weight earlier. Khachatourians reported that even low amount of antibiotics could encourage the selection of antibiotic-resistant gene (Khachatourians, 1998). However, these antibiotics-enhanced animal feeds often contain more than the recommended amounts (Dewey et al., 1997). Animals usually do not utilize all the antibiotics taken into their body. Significant proportions of antibiotics are excreted through urine or manure (Levy, 1992), which end up in the manure and manure effluent, thus affecting manure quality as a fertilizer. Once excreted, these antibiotics can enter the terrestrial environment through land application of manure and potentially alter the soil microbial ecosystem.

I.6 Traditional Treatment System

Animal farms handle a large amount of manure and it needs particular infrastructure and equipment such as storage site. In most cases in animal operations, composting and lagoon system are the most widely adopted methods to treat solid and liquid manure, respectively. The main purpose of liquid manure treatment is to break down organic matters, remove settleable solids, and reduce odor so that the treated effluent can be used for irrigation or washing and the

treated solids are suitable for land-application (Liu and Wang, 2020). Lagoon creates an anaerobic environment which enables bacteria and other organisms to degrade organic matters in the fluid. This process also allows most of the suspended solids in the liquid manure to settle as a *P*-rich sludge (Worley, 2007). As an alternative to anaerobic lagoon, facultative lagoon is also commonly used, which may achieve a closer to secondary treatment of liquid manure with enhanced oxygen supply. A facultative lagoon is typically designed with a depth shallower than that of an anaerobic lagoon.

Using lagoon treatment system provide many advantages such as low cost construction, minimum operating cost, reduced labor, maximum convenience in handling and land spreading of manure, compatibility with modern flush cleaning systems and pit overflow systems. There are some drawbacks, however, such as bad odors, significant loss of manure nitrogen content, a need of periodic sludge removal, and forming mosquito habitat. The most important thing is the lagoon is the final process before land application. There is no economically feasible method of further treatment and lack of strict regulation on lagoon effluent. Therefore, lagoons should be designed to fit local weather conditions and other environmental factors along with developing/applying practical method to reduce contaminants of final lagoon effluent.

In my dissertation for doctoral degree, I have focused on treating bacteria and antibiotics which can affect environment significantly. As treatment methods for these contaminants, zero-valent iron (ZVI) and cationic polymers such as PolyDADMAC, Polyamines, and Polyacrylamide, were chosen.

CHAPTER II

BACTERIAL INDICATOR REDUCTION IN DAIRY MANURE USING ACTIVE IRON MEDIA*

II.1 Introduction

Animal manure is a great nutrient source and soil conditioner for crop production. However, improper application of animal manure causes environmental issues such as microbial contamination or eutrophication through excess of nutrient (Islam et al. 2004; Ribaudo et al. 2003). In particular, applying manure with high levels of pathogens could pose health risks to animals and humans. Many studies reported that pathogen levels in manure can be reduced by chemical treatment using synthetic polymers (Liu et al. 2016) or biological treatment such as activated sludge process (Vanotti et al. 2005). There are numerous bacteria species present in manure with the most common pathogenic microorganisms found in manure being *Salmonella*, toxigenic *Escherichia coli* (*E. coli*), *Yersinia* and *Campylobacter* species, protozoa *Giardia*, and *Cryptosporidium* (Bicudo et al. 2003). Among those microorganisms, *E. coli* has been commonly used as an indicator organism of microbial contamination in a water system (S.C. Edberg et al. 2000).

Applying zero-valent iron (ZVI) to treat contaminated soils and groundwater have been becoming popular in the past two decades. Substantial experiences have been accumulated on kinetics of iron corrosion product/oxides and contaminant destruction. In addition, there also have been extensive researches on effect of ZVI performance, monitoring, modeling, and design (Blowes et al. 2000; Huang et al. 2003, 2013; Morrison et al. 2002; Shokes and Moller 2000).

*Reprinted with permission from "Bacterial indicator reduction in dairy manure using hybrid zero-valent iron (h-ZVI) system" by Sunghwa Han, Yongheng Huang, and Zong Liu, 2019. *Environmental Science and Pollution Research*, 26(11), 10790-10799, Copyright [2019] by Springer Nature BV

The known mechanism of removing contaminants by ZVI in aquatic system were reduction, precipitation, and adsorption. However, feasibility is the most challenging part, as applying ZVI into the real field is difficult because a surface passivation problem of ZVI in addition to the rapid loss of their reactivity by passivation. There have been thorough efforts to overcome the passivation problem. For example, addition of sand with iron can prevent iron passivation because contaminants compete with Fe (II) and Fe (III) generated from ZVI when they adsorbed onto sand; addition of MnO_2 can prevent passivation through redox reaction (Fe (II) generated from ZVI oxidation are used for reducing MnO_2 instead of producing iron corrosion products); activated carbon keeps ZVI reactivity as it is able to transfer electrons from iron corrosion product to contaminants. (Btatkeu-K et al. 2014; Dos Santos Coelho et al. 2008; Luo et al. 2014; Noubactep et al. 2011; Scherer et al. 2000; Shi et al. 2014).

In this study, we utilized a specific technique to prevent iron passivation problem by pre-treatment (preconditioning) of the ZVI with nitrate and ferrous iron as we developed in the previous study (Huang and Zhang 2005). The previous study demonstrated that magnetite was the dominant corrosion product as a result of iron-nitrate redox reaction (preconditioning). The reaction during the ZVI preconditioning was described as (Huang and Zhang 2005);

$$NO_3^- + 2.82\ Fe^0 + 0.75\ Fe^{2+} + 2.25\ H_2O \rightarrow NH_4^+ + 1.19\ Fe_3O_4 + 0.50\ OH^- \quad (1)$$

The strategy of augmenting the ZVI system with externally added Fe^{2+} to overcome ZVI surface passivation was highly effective (Huang et al. 2012, 2012, 2013). The preconditioning not only enhanced a reactivity of ZVI, but increased removal efficiency of contaminants without surface passivation. The previous study concluded that both Fe^{2+} and Fe_3O_4 (magnetite) generated from preconditioning process were important for pollutants degradation by ZVI (Huang et al. 2012, 2013; Tang et al. 2016). The effects of pre-treated ZVI (hybridized

ZVI/Fe_3O_4/Fe^{2+}, AIM) on removal of various contaminant in water systems have been demonstrated. However, very importantly, its effect against bacteria levels in wastewater has not been evaluated yet. Although some studies have reported study results of bactericidal effect of zero valent iron, most of the studies have focused on ZVI nano-particles (Diao and Yao 2009; Kim et al. 2010; Lee et al. 2008; Noubactep 2011; Xu et al. 2013). The effect of the pre-treated ZVI (AIM) in micron scale particle on bacterial removal has not been assessed yet.

Based on the understanding of the substantial roles of Fe^{2+} and magnetite in overcoming ZVI surface passivation and sustaining ZVI system's reactivity for contaminants reduction, we found that introducing the AIM into microbial contaminated aquatic system could effectively remove bacteria via adsorption and/or other possible mechanisms. The ultimate goals of this study were to: (1) estimate the effect of AIM system on bacterial attenuation, (2) demonstrate the bacterial removal mechanism, and (3) visualize the effects of AIM on bacteria. To achieve these objectives, we optimized the condition for ZVI preconditioning, modified reactors and designs for bacterial experiments, and conducted microscopic analysis for visualization. In addition, sonication method was used to determine the effect of AIM on adsorption. Our results from this study strongly suggests that AIM system can be used to treat water and wastewater where bacterial contamination is the major concern.

II.2 Material and Methods

II.2.1 Reagents

Zero valent iron (ZVI) grains of 20 μm (>99.2%, Johnson Matthey, UK) with a specific area of 0.690 m^2/g were used in this study. The ZVI characteristics were measured by BET nitrogen absorption analysis (Quantachrome, USA). All reagents for ZVI preconditioning were prepared with deoxygenated and deionized (DDI) water (E-pure D4641, USA) and stored in an anaerobic chamber. The DDI water was prepared by purging with N_2 for at least 30 minutes and stored in the anaerobic chamber for at least 24 hours to further remove residual O_2 before use. Fe^{2+} stock solutions (50 mM) were prepared with $FeCl_2 \cdot 4H_2O$ (J.T. Baker, USA) and stabilized by adding HCl (1 mM). Nitrate stock solution was prepared at 140 mM with $NaNO_3$ (Alfa Aesar, USA). Normal saline solution was prepared with 0.85% of NaCl (BDH, USA) in deionized (DI) water, followed by autoclave sterilization in 121°C for 15 minutes and stored in 4°C before use. All chemicals used in this study were analytical reagent grade.

II.2.2 *E. coli* isolation, identification, and preparation

E. coli was isolated from raw dairy manure collected from regional livestock research facility in central Texas. For bacteria isolation, 10 μL aliquot of raw manure was added to MacConkey agar media (Becton, Dickinson and Company, USA) using spread plate technique. The plates were incubated in 37°C for 24 hours. A single morphologically unique pink colony on the MacConkey media was transferred to a fresh plate by the streaking technique. A single colony from the second plate was transferred to MacConkey agar media to assure a pure bacteria strain was isolated. A physiological characterization of the bacteria was completed.

Quanti-tray method was used to qualitatively determine if the isolated strain is coliform and *E. coli* by manufacturer's instruction. A single pink colony was transferred to Luria-Bertani

(LB) broth media and incubated for 24 hours. The cultured suspension was serially diluted to 1/10,000 (v/v) using 99 mL of sterilized phosphate buffer saline (Hardy Diagnostics, USA) for Quanti-tray analysis. This method was also used to quantify bacterial concentration (total coliform and *E. coli*) of raw manure. The number of wells that showed color change after incubation were counted and compared with most probable number (MPN) table. The final concentration was showed in MPN/100 mL.

The samples of isolated strain were sent to the Laragen, Inc. (California, USA) for genotype analysis. 16S ribosomal RNA gene sequence (Appendix I) was obtained and the result was compared with NCBI nucleotide BLAST search. The isolate was determined to have a high (~99%) 16S rRNA sequence similarity to *E. coli* strain NBRC 102203. The isolated bacteria was sub-cultured daily on the Tryptic Soy Agar (TSA, Becton, Dickinson and Company, USA) media. Bacterial stock was prepared with 60% sterilized glycerol and stored at -20°C for future use.

For activation and enumeration, single colony of *E. coli* from the plate was collected and transferred to Tryptic Soy Broth (TSB) media and incubated at 37°C for at least 16 hours before each set of experiments. Bacterial concentration was determined by measuring optical density with spectrophotometer at wavelength of 600 nm (1.0 at OD_{600} equals to 8×10^8 colony forming unit (CFU)/mL). The bacterial suspension was serially diluted and inoculated into the reactor. Final concentration of bacteria in the reactor was 5~10 $\times 10^6$ CFU/mL.

II.2.3 AIM preparation

15-mL centrifuge tubes were used as the reactor for preparing the activated iron media. As the first step, 0.5 ± 0.002 g of ZVI was added into the reactors, which was then transferred into the anaerobic chamber. Fe^{2+} and NO_3^- stock solution of designed volumes and DDI water

were pipetted into the reactor to achieve a reactant solution of 10 mL in total (7 mM Fe^{2+} and 10 mM NO_3^-). The reactors were sealed with a cap in the anaerobic chamber and then transferred into a rotary tumbler for complete mixing at 30 rpm at 25 $\pm$ 2°C for 17 h in the dark.

II.2.4 Bacterial reduction experiment

The bacterial reduction experiment was also conducted in 15-mL centrifuge tube (reactor). After 17 hours of preconditioning, the remaining liquid was separated using a magnet and discarded. The prepared AIM was then washed three times with DI water. Fe^{2+} was taken from prepared stock solution in an anaerobic chamber and added to the reactor at a desired concentration (0.1 mM) with an activated bacterial suspension concentration of 5~10 × 10^6 CFU/mL. Total volume of the reactors was 10 mL. Subsequently, reactors were transferred and laid horizontally on the shaker. The cap of the reactor was not fully tightened for aeration. Reactors were shaken at 200 revolutions per minute (rpm) for desired reaction time. After reaction, the bulk phase (liquid phase) of each reactor was separated from AIM media using a magnet and collected to a fresh sterile bottle. Collected samples were serially diluted with normal saline solution and the live cell concentration was measured by the pour plate method. The remaining AIM solids were used to evaluate the concentration of attached bacteria. The solid media was washed three times with normal saline solution, then filled with the same volume (10 mL) as an initial volume. Each reactor was sonicated (Bransonic 220, USA) for 30 seconds to desorb the attached *E. coli.* The supernatant of sonicated reactors was then collected and used to evaluate desorbed *E. coli*. In order to avoid any possible interference and osmotic stress to the bacteria, all experiments were performed in normal saline solution. The bacteria reduction experiments were all performed in duplicate and repeated three times.

II.2.5 Microscopic analysis

Fluorescence microscopy (Olympus BX41, USA) was used to visualize the effects of AIM media against *E. coli* viability. *E. coli* culture suspension was diluted with normal saline solution for CTC-DAPI double staining according to a modified technique of Coleman and Rodriguez (Coleman 1980; Rodriguez et al. 1992). 5-cyano-2,3-di-(p-tolyl) tetrazolium chloride (CTC, Sigma-Aldrich) and DNA-binding fluorochrome 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) were used as a staining dye for live and total cells, respectively. 10 ppm of CTC (final concentration) was added to bacterial culture suspension and incubated for 3 hours at room temperature while shaking (100 rpm) in the dark. DAPI counterstaining dye was added after incubation. Counterstaining with DAPI allowed concurrent determination of total (i.e., viable plus nonviable) and respiring (i.e., cells exhibiting CTC-formazan fluorescence) cell counts in a single preparation. The stained bacterial cell was treated with the method described at section 2.4 for desired contact time. Observations were conducted with a fluorescence light fitted with a 385 nm excitation filter and a 455-nm dichroic mirror, allowing visualization of each dye. The emission wavelength was 420 nm and 580 nm for CTC and DAPI, respectively.

Transmission electron microscope (JEOL 1200 EX, USA) was used to observe whether the bacterial membrane damage occurred. Both of treated and untreated samples were prepared and negatively stained for TEM analysis. For treated sample, both bulk and solid phase were observed to visualize the effect of AIM on *E. coli*. After 2 hours of reaction, 2 µL of each sample was collected and applied onto the observation grids (400 mesh copper grids with carbon support film). Subsequently, 2% aqueous uranyl acetate solution was applied to each of the sample grid. The samples were stored at room temperature for 15 minutes to air dry and were then observed.

II.2.6 Statistical analysis

Experiments were performed at least in triplicate throughout the study and all data are presented as the mean ± standard error of repeated values. Statistical significance was determined using Student's t-tests by evaluation of differences between treated and control groups; $*p < 0.05$ or $**p < 0.01$ were considered statistically significant based on the tests. JMP pro 13 was used as a tool for statistical analysis.

II.3 Results and Discussion

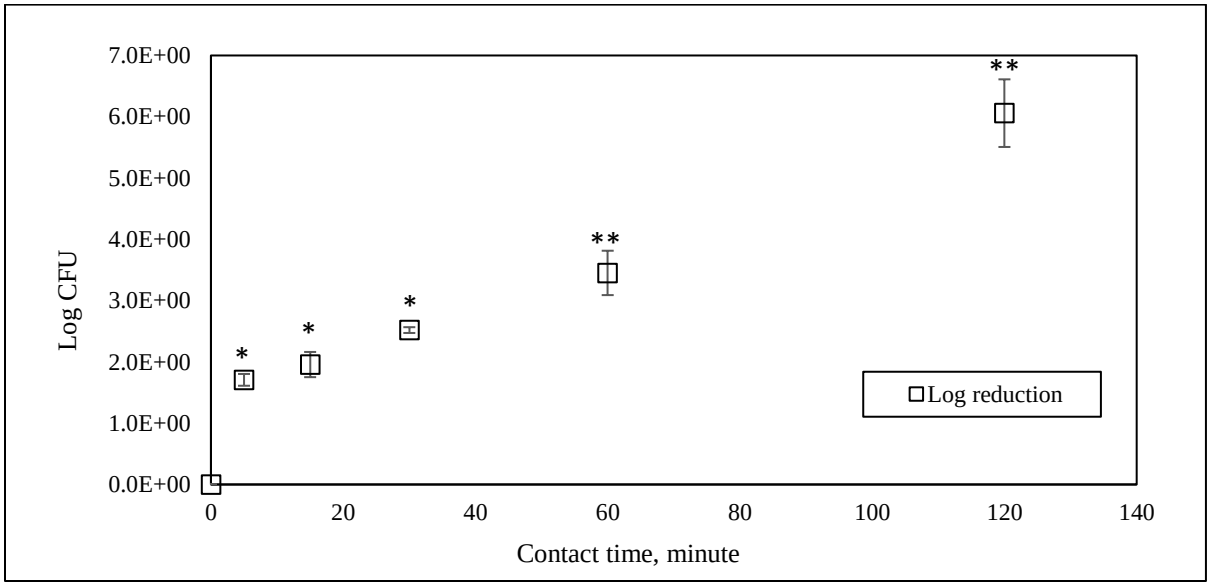
II.3.1 ZVI preconditioning

During the ZVI preconditioning process, all nitrate played a role as an electron acceptor and completely reduced to ammonia. The nitrate resulted in aqueous Fe^{2+} disappearance from solution and formation of a black oxide coating (magnetite). As long as Fe^{2+} is available in the aqueous phase, magnetite (Fe_3O_4) could be formed and coated onto the surface of ZVI (Huang and Zhang 2005; Huang et al. 2003; Tang et al. 2016). The actual amount of Fe_3O_4 produced from the preconditioning could be slightly higher due to the presence of other reducible compounds such as dissolved oxygen (Huang et al. 2013). The primary role of Fe^{2+} in the system was to facilitate ferric (hydr)oxides, the dominant passive corrosion products, transformation to magnetite. It was also demonstrated by previous studies that externally supplied Fe^{2+} can overcome ZVI surface passivation (Huang et al. 2012, 2013; Tang et al. 2016).

II.3.2 Effects of AIM on *E. coli* removal

The effects of AIM on *E. coli* removal is shown in Fig. 1-a). *E. coli* concentration in the bulk liquid phase of the reactor was gradually decreased with reaction time and non-detected after 2 hours of reaction. Bacterial suspension in normal saline solution with and without Fe^{2+} was evaluated as a control and blank, respectively.

(a)



(b)

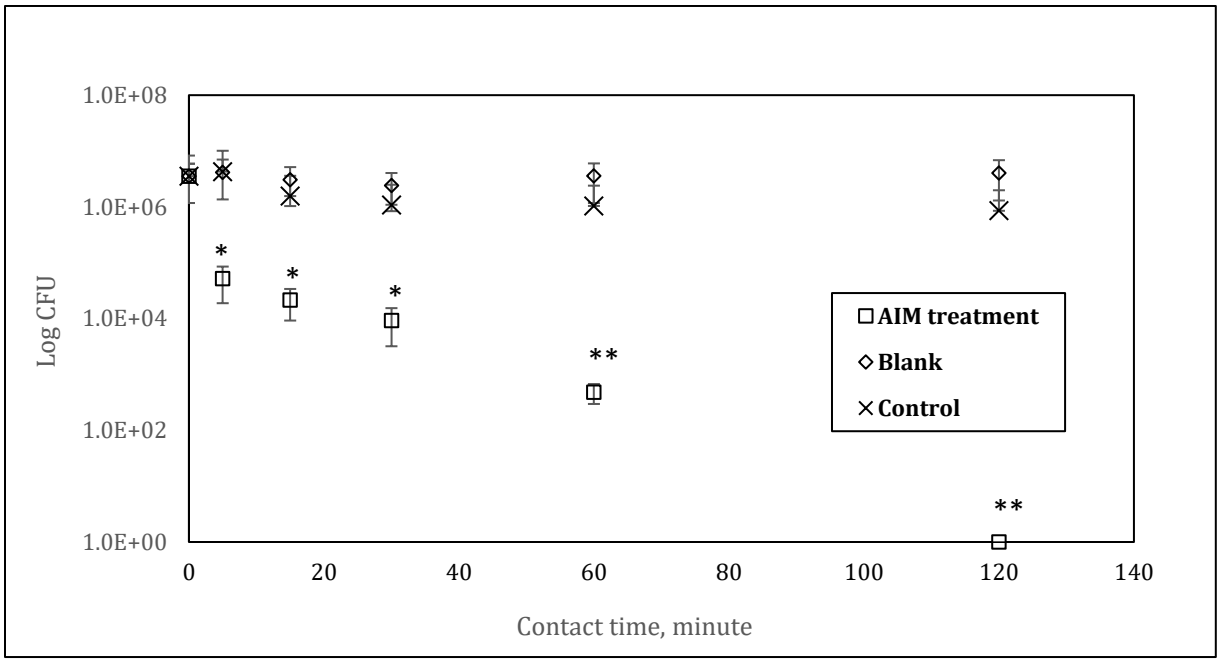


Figure 1. Effects of AIM and Fe^{2+} on *E. coli* removal in bulk phase: (a) Log reduction by AIM media; (b) Log reduction by blank (cell only) and control (0.1 mM Fe^{2+} only).

The mechanism of *E. coli* removal by AIM is likely a combination of adsorption and inactivation. The concentration of live bacterial cells adsorbed to the media decreased with time, which suggests that the adsorption is dominant on bacterial removal in earlier phase of the reaction, followed by inactivation. (Fig. 2). The control and blank tests showed that 0.1 mM of Fe^{2+} had limited effect on *E. coli* viability within 2 hours of reaction (Fig. 1-b). According to the control and blank tests, we could conclude that AIM, not Fe^{2+}, is the major reason for *E. coli* removal in the system.

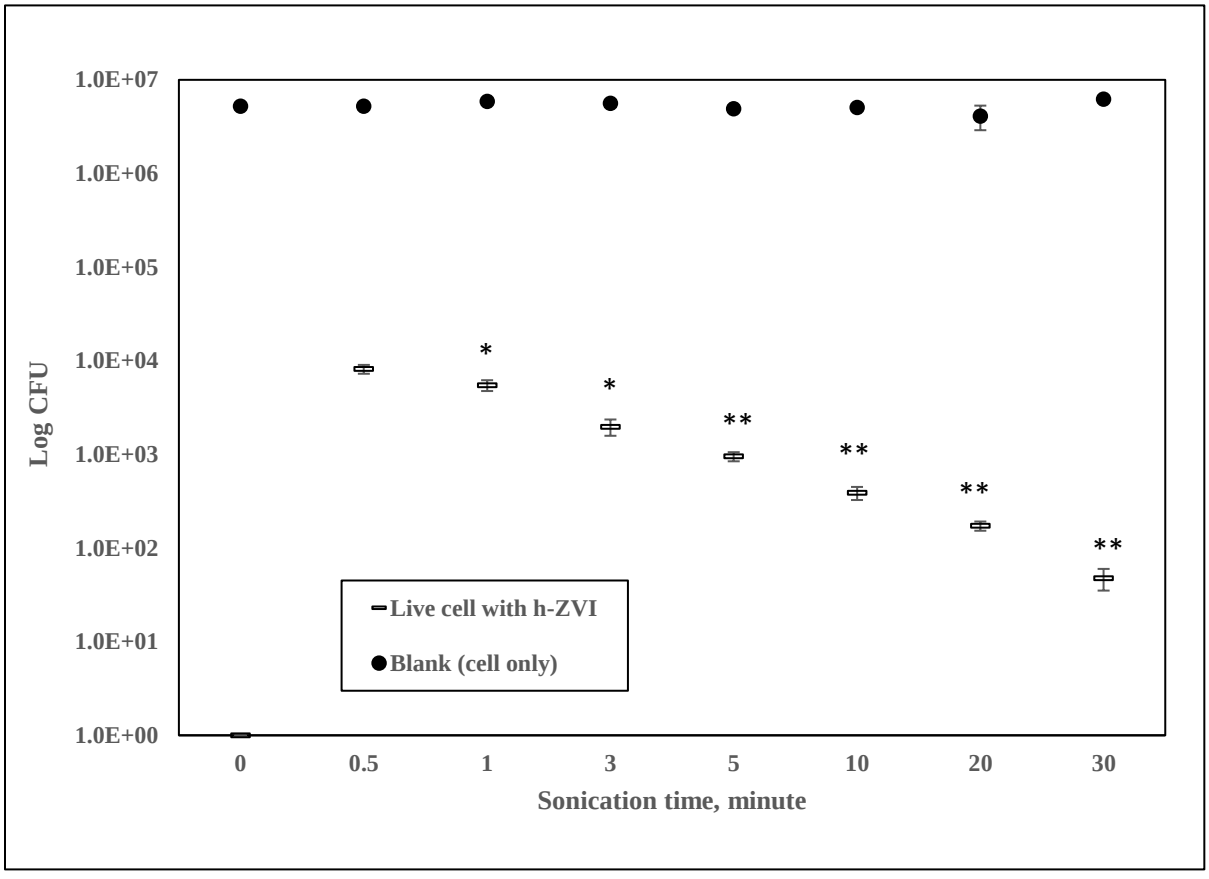


Figure 2. Effect of sonication on *E. coli* viability with or without adsorption.

Magnetite has a natural surface affinity for bacteria (Latour and Kolm 1976; Macrae and Evans 1983), but has limited effect on bacterial viability (< 0.1 log reduction) against *E. coli* (Lee et al. 2008). Therefore, the magnetite generated from ZVI preconditioning may play a key role in decreasing bacterial population via adsorptive immobilization from the bulk liquid system upon contact with media. A major factor in the adsorption of bacteria by magnetite is the positive surface charge on the magnetite particles at pH values lower than the isoelectric point (IEP). The IEP of magnetite is known as 7.9 (Tombácz et al. 2006). Typical gram negative bacteria including *E. coli* have been reported to have an IEP values between 2.0 to 3.0, which means that the bacteria should have a negatively charged cell surface in neutral pH (Rijnaarts et al. 1994). The zeta potential of *E. coli* cell particles in the system after reaction gradually decreased (getting more negative) with reaction time (Fig. 3), which could support the ability of magnetite on bacterial adsorption in the system.

The effect of AIM against total coliform and *E. coli* in raw dairy manure was also evaluated. The removal efficiency of AIM on total coliform and *E. coli* significantly decreased in raw manure compared to the experimental condition (normal saline solution) used in this study. The removal efficiency of AIM against total coliform and *E. coli* were 92.44% and 88.12%, respectively (Fig. 4), when AIM was applied to raw manure. This is significantly lower bacterial removal efficiency compare to *E. coli* removal in a normal saline solution. The possible reasons of this discrepancy could be attributed to the presence of natural organic matters (NOM) and nutrients in the raw manure. Chen et. al demonstrated the effect of NOM on nano scale ZVI reactivity and the reactivity significantly decreased by NOM because of occlusion of reactive site on the surface of ZVI (Chen et al. 2011, Redman et al. 2002).

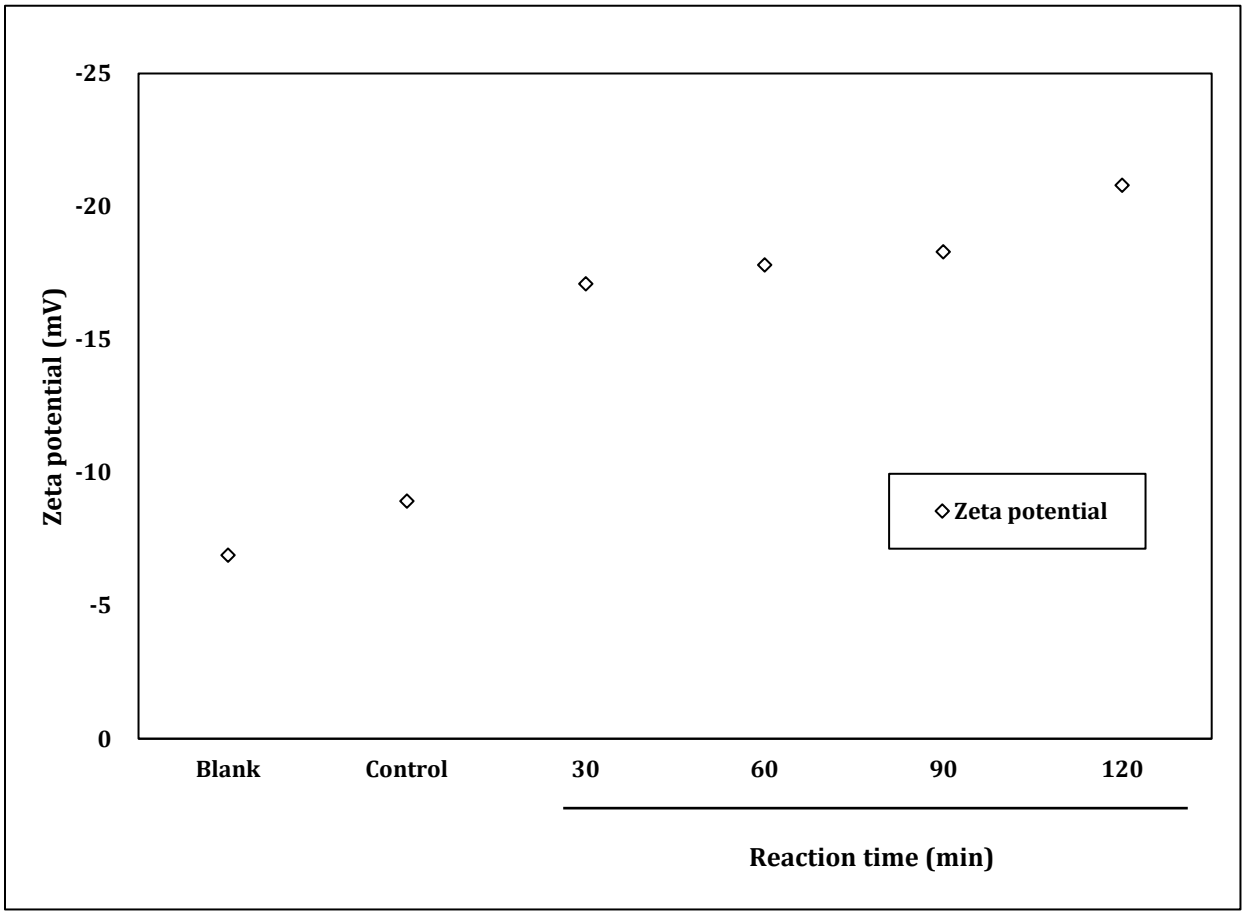


Figure 3. Change of zeta potential of *E. coli* particle with contact time. Blank: *E. coli* in normal saline solution; Control: *E. coli* in normal saline solution with 0.1 mM Fe^{2+}.

Another factor that can affect the bacterial removal efficiency is nutrients source in water samples. Raw manure is rich in bacterial growth nutrient such as organic carbon, nitrogen, phosphorus as well as potassium, calcium, magnesium, and other essential elements (Payne and Lawrence 2015). In addition, a previous study assessed the effect of manure on microbial growth, the results demonstrated dairy manure is a suitable source for microbial activities (Liu et al. 2016).

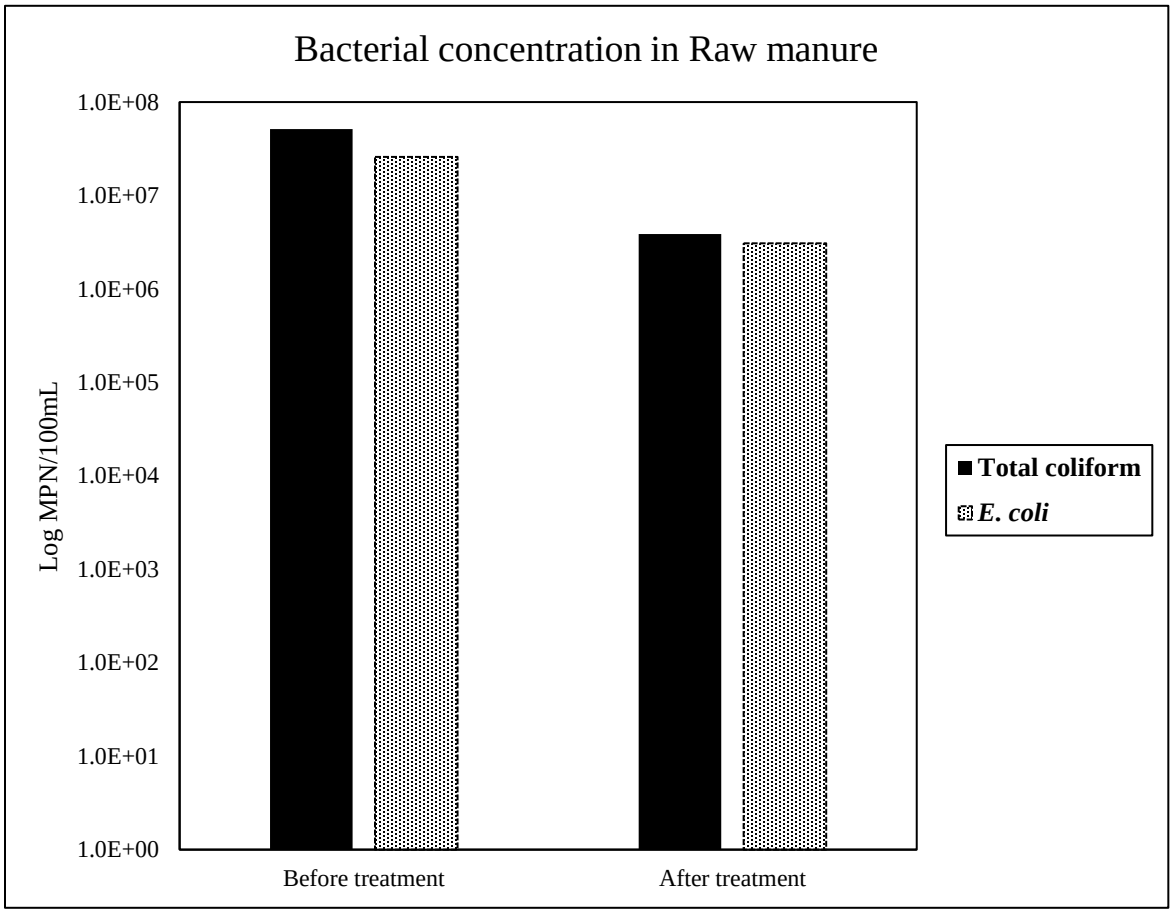


Figure 4. Effect of AIM on total coliform and *E. coli* of raw manure.

In particular, dairy manure contains high level of phosphorus, which is a critical element for microbial growth. Therefore, using manure can greatly promote microbial viability (Miettinen et al. 1997). Soluble phosphorus can react with aqueous Fe^{2+} and be precipitated very rapidly. It could be inferred that lack of Fe^{2+} in the system caused the inhibition of electron transfer from ZVI, which leads the suppression of ZVI reactivity (Huang and Zhang 2005). The average concentration of soluble phosphorus in the manure sample was measured 39.12 mg/L as

P which exceed the capacity of externally added Fe^{2+} (0.1 mM), resulted in reduction of removal efficiency. Other possible mechanisms that could affect the reaction between AIM and bacterial removal are not investigated in this study.

II.3.3 Effects of AIM on bacterial adsorption

After reaction, sonication was applied on remaining AIM media (solid phase) in order to evaluate the mechanism of bacterial removal by AIM media. To determine the effect of sonication on *E. coli* attachment, the final concentration of *E. coli* in the reactor was measured after desired time of sonication was applied. The minimum contact time (5 minutes of contact) was chosen in order to minimize possible reactions which affects the bacterial viability. Sonication of bacteria without AIM media was also performed as a control (Fig. 2). The live *E. coli* concentration decreased with sonication time (Fig. 2) because the bactericidal reaction on *E. coli* occurred when AIM media presented during sonication. The longer sonication time, the greater chance of reaction between the media and bacteria, which would result in inhibition of bacterial viability. Therefore, we concluded the optimal sonication duration to be 30 seconds for the desorption experiment. In addition, results of control experiment confirmed that 60 Hz of sonication without AIM media does not affect *E. coli* viability until 30 minutes (Fig. 2).

Fig. 5 showed the live *E. coli* concentration after sonication by contact time. As expected, the live *E. coli* concentration adsorbed to the media decreased with increase of contact time. At the contact time of 120 minutes, the live cells were completely removed/inactivated in the bulk phase, but adsorbed cells were not completely inactivated. Therefore, these results imply that the adsorption onto the surface of AIM media is dominant mechanism of bacterial removal in earlier phase of reaction (< 5 minutes) and bacterial inactivation by the AIM media is a slower process compare to the adsorption.

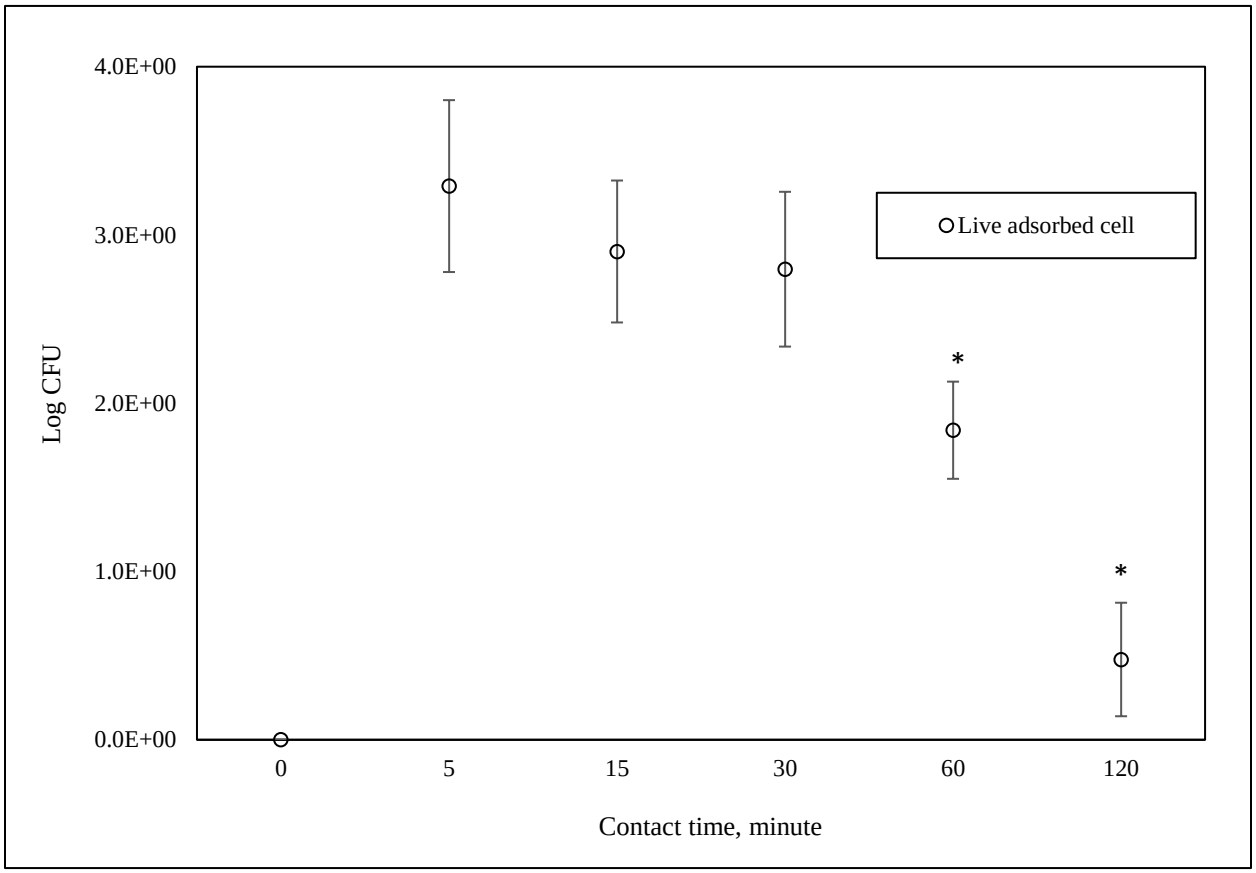


Figure 5. Effect of contact time on *E. coli* viability.

II.3.4 Microscopic analysis

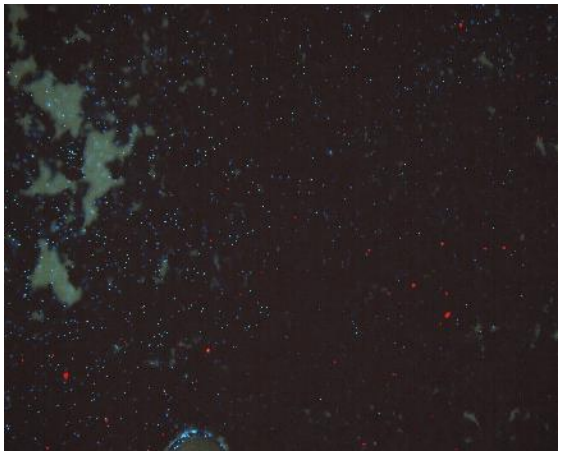
The interactions between *E. coli* cells and AIM media were visualized by fluorescence microscopic analysis. The results of *E. coli* adsorption and inactivation by AIM media were shown in Fig. 6 and Fig. 7. Fig. 6-a, b, and c showed the CTC-DAPI double stained fluorescence microscopic images of *E. coli* after 5, 30, and 2 hours of reaction, respectively. We could confirm from this image analysis that the adsorption was a rapid process. Majority of the cells were adsorbed within 5 minutes of reaction (Fig. 6-a). The decreasing red fluorescence in Fig. 6

can be interpreted as the *E. coli* was quickly adsorbed onto the surface of AIM media, followed by inactivated via redox reaction of AIM media. The longer reaction time, the less red fluorescence was emitted from CTC formazan, which is also consistent with the result of sonication desorption experiment.

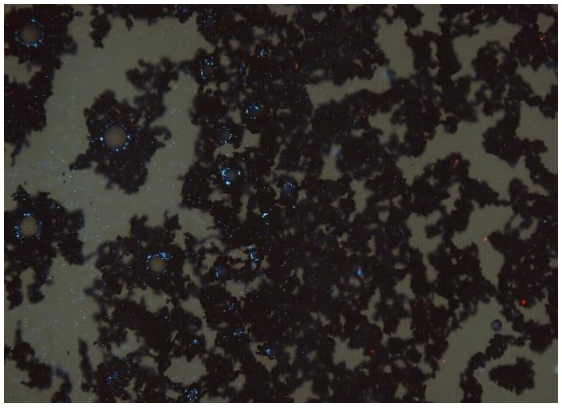
The fluorescence microscopic images suggest that adsorption was not the only mechanism responsible for bacterial removal in the AIM system. During the reaction, ZVI of the AIM system was oxidized, cytotoxic substances such as reactive oxygen species (ROS) were possibly produced from the media and affected bacterial cell viability (Keenan et al. 2009; Keenan and Sedlak 2008). The effect of AIM on bacterial inactivation was observed by TEM analysis.

The samples for TEM analysis was divided into untreated (control) group and AIM treated group; the treated group was again divided into the bulk liquid phase and the solid media phase. The liquid phase was evaluated to determine whether bacterial inactivation could occur without adsorption to the media or not, while the solid phase was collected for observing cell activity and condition after adsorbed onto the media. *E. coli* cells from the control group showed intact shape without damage of cell membrane (Fig. 8-a). Comparing with control group, the TEM images of solid phase of treated group confirmed that the membrane of *E. coli* cells adsorbed to AIM media were damaged or destroyed (Fig. 8-b).

a) 5 minutes of contact



b) 30 minutes of contact



c) 120 minutes of contact

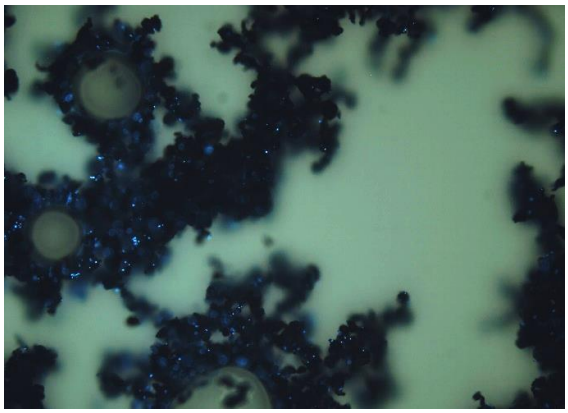
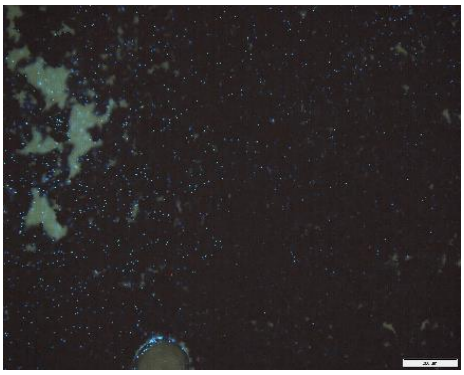
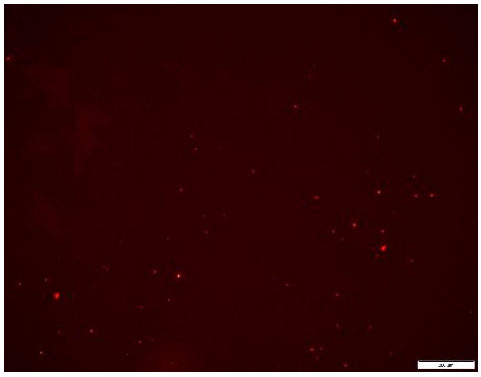


Figure 6. Overlapped CTC-DAPI double stained fluorescence microscopic images in different contact time: a) 5 minutes; b) 30 minutes; and c) 120 minutes of contact time.

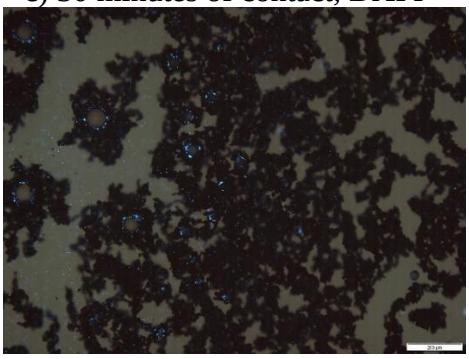
(a) 5 minutes of contact, DAPI



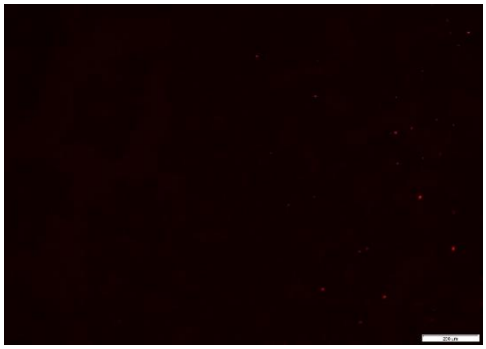
b) 5 minutes of contact, CTC



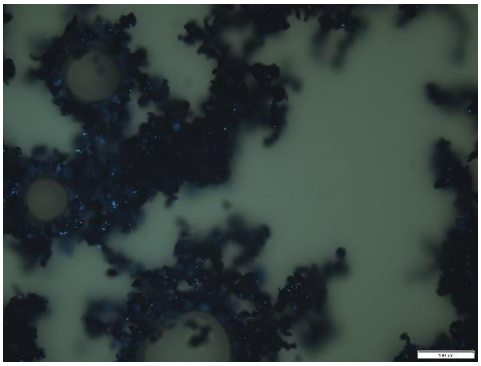
c) 30 minutes of contact, DAPI



d) 30 minutes of contact, CTC



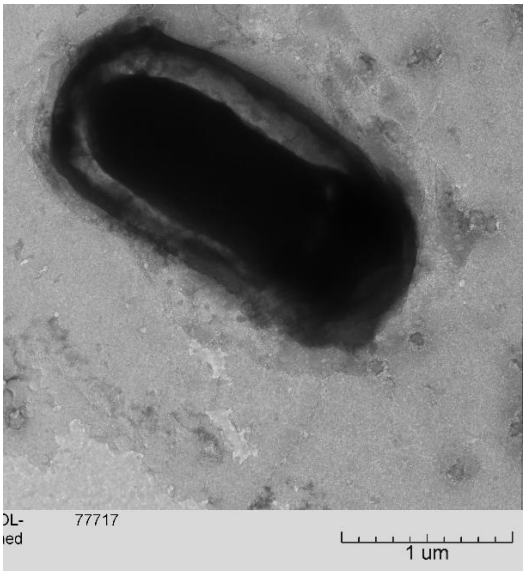
e) 120 minutes of contact, DAPI



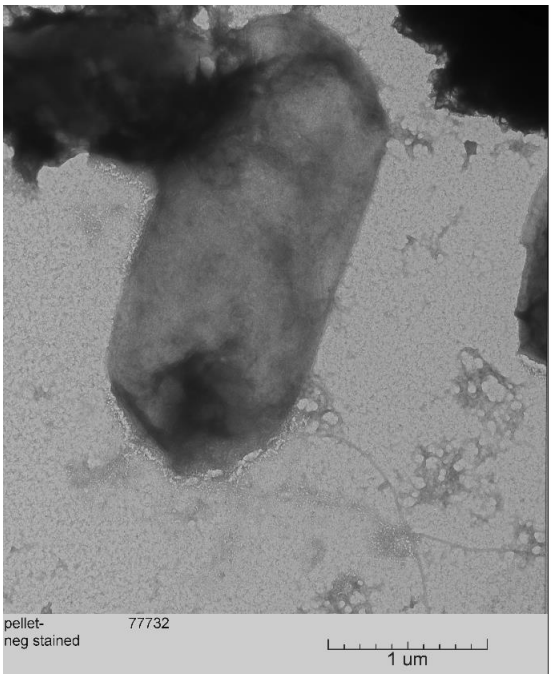
f) 120 minutes of contact, CTC

Figure 7. CTC-DAPI double stained fluorescence microscopic images in different contact time: a) 5 minutes; b) 30 minutes; c) 120 minutes of contact time.

a) control



b) Solid phase



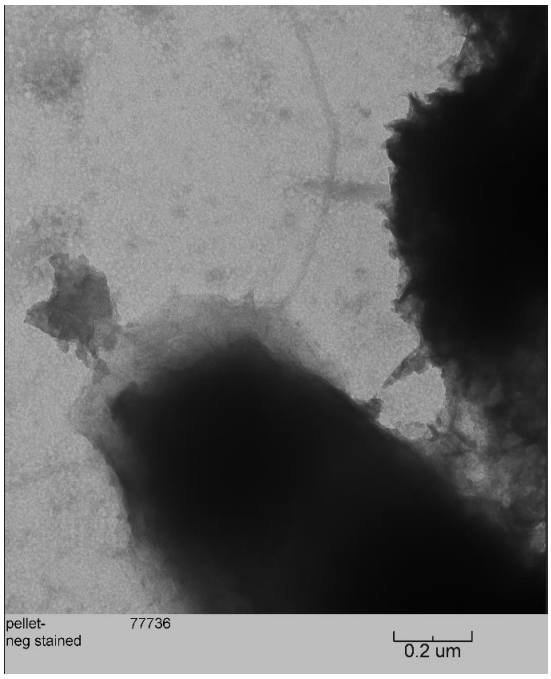


Figure 8. Negatively stained TEM image of a) intact *E. coli* cell; and b) solid phase after 120 minutes of contact time.

As shown in the TEM image, AIM was found to inactivate *E. coli* within 2 hours by direct membrane damage which is likely caused by oxidation of iron. However, we could not observe inactivated *E. coli* cells in the bulk phase from TEM analysis (Fig. 9) because most of the cells were adsorbed onto the media. However, flagella fragments were found in the TEM images of bulk phase (Fig. 9), which can be an evidence of cell surface damage and destroyed membrane. The result of previous study by Kim et al. stated that the bactericidal performance of ZVI does not occur in the bulk phase (Kim et al. 2010). Therefore, we can conclude that direct contact between cell and media should be responsible for cell death via certain reactions inside the cell or the direct damage of the cell membrane through the ROS generation.

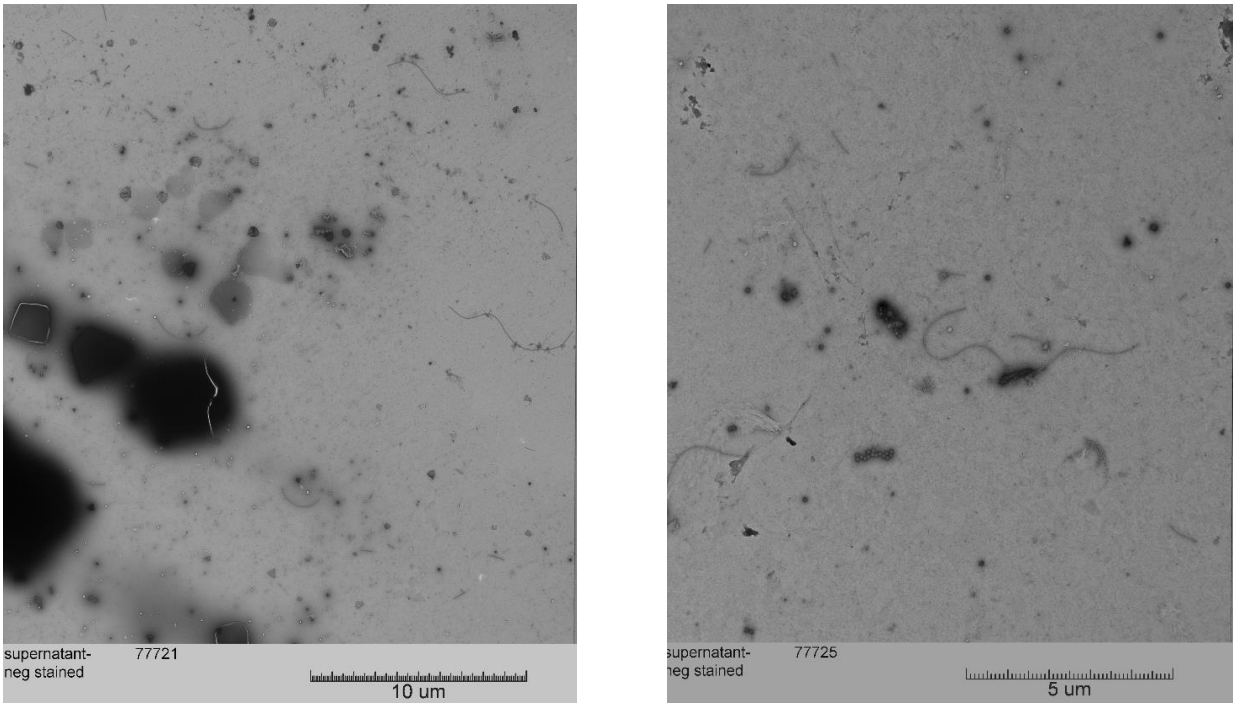


Figure 9. Negatively stained TEM image of bulk phase after 120 minutes of contact time.

These results suggest that bacterial can be removed not only by adsorption, but also by inactivation from AIM media oxidation. It is believed that the dominant mechanism responsible for bacteria inactivation is ROS generation. Previous studies have reported that ROS such as superoxide or hydrogen peroxide can be produced by oxidation of ZVI nano particle. The introduction of ZVI or Fe^{2+} to the system could produce a rapid burst of an oxidant. Superoxide and hydrogen peroxide were produced as the ZVI was oxidized (Keenan et al. 2009). Although the ZVI used in this study was not a nano scale particle, previous studies have demonstrated that the AIM media had strong redox capacity (Huang and Zhang 2005). The AIM could act as a strong reductant so that it significantly affects *E. coli* viability. In the AIM system, the ZVI, not Fe^{2+}, plays a major role in a redox reaction, which means that ZVI is responsible for bacterial inactivation (Huang and Zhang 2005; Tang et al. 2016).

Dissolved oxygen (DO) in the reactor can also affect bacterial removal efficiency. Lee et al. (2008) reported that a strong bactericidal effect of nano ZVI was found in the absence of oxygen. Previous study from our group demonstrated that AIM media can rapidly consume DO, result in anoxic environment at neutral pH condition (Huang and Zhang 2005). Although the mechanism for the bactericidal reaction of ZVI or Fe (II) is not yet fully understood, the presence of Fe (II) with ZVI likely induced oxidative stress by generating ROS through the Fenton reaction (Touati 2000). The reaction of the oxidant producing mechanism can be simplified as following reaction (Keenan et al. 2009):

$$Fe^0_{(s)} + O_2 + 2H^+ \rightarrow Fe\ (II) + H_2O_2 \quad (2)$$

$$Fe^0_{(s)} + H_2O_2 + 2H^+ \rightarrow Fe\ (II) + 2H_2O \quad (3)$$

$$Fe\ (II) + O_2 \rightarrow Fe\ (III) + O_2^{-\cdot} \quad (4)$$

$$Fe\ (II) + \ O_2^{-\cdot} + 2H^+ \rightarrow Fe\ (III) + \ H_2O_2 \quad (5)$$

$$Fe\ (II) + \ H_2O_2 \ \rightarrow oxidant \quad (6)$$

Based on the information from the previous study, we postulated that bacteria might be inactivated by oxidative stress during ROS generation. Following-up studies of our group will be conducted to determine which ROS species are directly responsible for the bacterial inactivation in the AIM media system.

II.4 Implication

This study is the first attempt to discover AIM's impact on bacterial removal, especially in animal wastewater. AIM has been widely used for wastewater treatment facilities, mainly focuses on heavy metal removal by its redox capacity. AIM technology has suggested effective solution to overcome the problems of normal ZVI has such as passivation. The highlight of this study is to evaluate the extended ability of AIM media on removal and inactivation of fecal bacterial indicator. In addition, particle size of ZVI used in this study was micron scaled particle which is more feasible in terms of field scale application comparing to ZVI nanoparticles. As shown in Fig. 4, high concentration of fecal indicator presents in raw manure, and it's a potential risk for disease outbreaks animal herds offsite users (Liu et al. 2015). Therefore, appropriate and cost-effective treatment method is one of the most important things in agricultural wastewater treatment and the industry have always required proper technology. This study demonstrated that AIM can be a supplement treatment at manure or wastewater treatment facilities where pathogen contamination is concerned.

II.5 Conclusion

This study suggests that AIM can significantly reduce fecal indicator organism. The following conclusions can be drawn:

- ZVI is coated with magnetite (Fe_3O_4) by preconditioning, resulted in preventing passivation of ZVI.
- Magnetite is responsible for adsorptive removal of fecal indicator, but does not affect bacterial viability.
- Adsorption between AIM media and bacteria is very rapid process and inactivation is occurred after adsorption by iron oxidation.
- Fluorescence and electron microscopic images confirm the effects of AIM on bacterial removal and inactivation in contact time dependent manner.
- AIM system can be used for wastewater treatment facilities where microbial contamination is concerned such as manure effluent.

II.6 Summary

Novel and efficient animal wastewater treatment technologies of bacteria reduction are needed for preventing disease outbreak in animal herds and safeguarding environmental health. Zero-valent iron (ZVI) has been used to treat bacterial contaminated water for past decades, but its passivation issue has been a major challenge. In this study, batch tests were performed to evaluate the effect of AIM or a mixed ZVI/Fe_3O_4 media system on reduction of *Escherichia coli* (*E. coli*) levels. The AIM media was created through a wet chemical process that uses nitrate to oxidize ZVI in the presence of externally added Fe^{2+} (aq.). Transforming ZVI into a AIM system could overcome the passivation of ZVI and increase the reactivity of the media. The results demonstrated that *E. coli* cells in the bulk phase were removed rapidly by AIM media. Majority of *E. coli* was attached (or adsorbed) to the surface of AIM media within a few minutes, which suggested that adsorption was the dominant mechanism for bacterial removal in initial phase. This adsorption was confirmed by fluorescence microscopy with CTC-DAPI double staining and transmission electron microscopic (TEM). Increasing contact time steadily inactivated of *E. coli*; all cells were inactivated after 120 minutes of contact. The TEM results indicated that AIM inactivated *E. coli* by causing direct damage on bacterial cell membrane. The results of this study strongly suggest that AIM treatment can be used in water treatment industry where bacterial contamination is concerned.

CHAPTER III

STUDY ON THE BACTERIAL INACTIVATION MECHANISM OF ACTIVE IRON MEDIA

III.1 Introduction

Applying zero-valent iron (ZVI) to treat contaminated soils and groundwater have been becoming popular in the past two decades. Substantial experiences have been accumulated on kinetics of iron corrosion product/oxides, contaminant destruction, and their effects on performance, monitoring, modeling, and design and extensive researches have been assessed to investigate degradations of a variety of contaminants by ZVI (Blowes et al. 2000; Huang et al. 2003; Huang et al. 2013; Morrison et al. 2002; Shokes and Moller 2000).

Several studies have reported the inactivation of microorganisms using iron-based media such as iron oxide, micro-sized iron granules, and nano particulate zero-valent iron (n-ZVI, Fe^0) (Lee et al. 2008; Kim et al. 2010). Several mechanisms may contribute to the antimicrobial activity of iron-based compounds, including oxidative stress from the reactive oxygen species (ROS) generated by the reaction of these compounds with O_2 or H_2O_2, as well as direct physical and chemical interactions between the compounds and the organisms (Dayem et al. 2017).

Fenton's reagent (i.e., Fe^{2+}/H_2O_2) is known to produce oxidants which are capable of oxidizing organic compounds in aqueous solution. Iron, as a partner of the Fenton reaction, potentiates oxygen toxicity (Touati 2000). Strict regulation of iron metabolism, and its coupling with regulation of defenses against oxidative stress, is an essential factor for living organisms in the presence of oxygen. Despite the fact that ROS, such as singlet oxygen, superoxide anion,

hydrogen peroxide, and hydroxyl radical, resulting from the transfer of energy or electrons to oxygen, are essential intermediates in certain physiological processes (e.g., photosynthesis, respiration, and cell signaling), and their levels within cells are well controlled via enzymes or antioxidants, exposure to high level of these ROS can be potentially harmful to living organisms (Ray et al. 2012). There are some known mechanisms of ROS generation from ZVI including homogenous reaction, non-radical mechanism for the homogeneous Fenton's reaction, the Haber-Weiss reaction, homogeneous Fe^{2+} autooxidation, and homogeneous auto-scavenging reaction (Wu et al. 2014).

Mixed ZVI/Fe_3O_4 media or hybrid ZVI (AIM) media is a specific technique to prevent passivation problem by pre-treatment (or preconditioning) of the ZVI with nitrate and ferrous iron. The strategy of augmenting the ZVI system with externally added Fe^{2+} to overcome ZVI surface passivation was highly effective (Huang et al. 2003; Huang, et al. 2013; Huang et al. 2012). The preconditioning of ZVI not only enhanced a reactivity of ZVI, but also increased removal efficiency of contaminants without surface passivation issues. It has been concluded that both Fe^{2+} and magnetite (Fe_3O_4) generated from preconditioning process were very important for pollutants degradation by ZVI (Huang et al. 2013; Huang et al. 2012; Tang et al. 2016).
In the previous research, we demonstrated the effect of mixed ZVI/Fe_3O_4 media or hybrid ZVI media (AIM) on removal of *Escherichia coli* (*E. coli*) isolated from raw manure in aquatic condition (Han et al. 2019). This study is a continuing study; the objective of current study is to investigate the bacterial inactivation mechanism of AIM against *E. coli*.

III.2 Materials and Methods

III.2.1 Reagents

Zero valent iron (ZVI) grains of 5 μm (> 99.2%, Johnson Matthey, UK) was used in this study. All reagents for ZVI preconditioning were prepared with deoxygenated and deionized (DDI) water (E-pure D4641, USA) and stored in an anaerobic chamber. The DDI water was prepared by purging with N_2 gas for at least 30 minutes and stored in the anaerobic chamber for at least 24 hours (h) to further remove residual O_2 before use. Fe^{2+} stock solutions (50 mM) were prepared with $FeCl_2 \cdot 4H_2O$ (J.T. Baker, USA) and stabilized by adding HCl (1 mM). Nitrate stock solution was prepared at 140 mM with $NaNO_3$ (Alfa Aesar, USA). Normal saline solution was prepared with 0.85% of $NaCl$ (BDH, USA) in deionized (DI) water, followed by autoclave sterilization in 121°C for 15 minutes and stored in 4°C before use. All chemicals used in this study were analytical reagent grade.

Dimethyl sulfoxide (DMSO) (J.T. Baker, USA), sodium azide (SA) (Amresco, USA), and Manganese (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) chloride (Calbiochem, USA) were used as a specific scavenger for hydroxyl radical, singlet oxygen, and superoxide anion, respectively. Stock solution of each reagent was prepared with deionized (DI) water in a concentration of 10 mM, 2.8 M, 10 mM, and 1 mM, respectively. MnTBAP chloride stock solution was stored at -20°C by manufacturer's instruction, and other three are stored at 4°C before use.

III.2.2 *E. coli* isolation, identification, and preparation

E. coli was isolated from raw dairy manure collected from regional livestock research facility in central Texas. The isolation and identification were conducted in the previous study[14] and the detailed procedure is described in the material part of Chapter II. The isolated bacteria

was sub-cultured daily on the Trytic Soy Agar (TSA) (Becton, Dickinson and Company, USA) media. Bacterial stock was prepared with 60% sterilized glycerol and stored at -20°C for future use. For activation and enumeration of isolated *E. coli*, single colony from the agar plate was collected and transferred to Tryptic Soy Broth (TSB) media and incubated at 37°C for at least 16 h before each set of experiments. Bacterial concentration was determined by measuring optical density with spectrophotometer at wavelength of 600 nm (1.0 at OD_{600} equals to 8×10^8 colony forming unit (CFU)/mL). The bacterial suspension was serially diluted and inoculated into the reactor. Final concentration of bacteria in the reactor was 0.5~1.0 × 10^6 CFU/mL.

III.2.3 AIM preparation (preconditioning of ZVI)

15-mL centrifuge tubes with cap were used as the reactor for preparing the activated iron media. As the first step, 0.5±0.002 g of ZVI was added into the reactors, which was then transferred into the anaerobic chamber. Desired volumes of Fe^{2+}, NO_3^-, and DDI water were pipetted into the reactor to achieve a reactant solution of 10 mL in total (7 mM Fe^{2+} and 10 mM NO_3^-). The reactors were sealed with cap to in the anaerobic chamber to block oxygen, and then transferred into a rotary tumbler for complete mixing at 30 rpm at 25 $\pm$ 2°C for 17 h in the dark.

III.2.4 ROS scavenger assay

After preconditioning, the remaining liquid was separated discarded using a magnet. The prepared AIM was then washed three times with DI water. Fe^{2+} and each scavenger were taken from prepared stock solution and added to the reactor at a desired concentration with activated bacterial suspension concentration of 0.5~1.0 × 10^6 CFU/mL. Total volume of the reactors was 10 mL. Subsequently, reactors were transferred and laid horizontally on a shaker. The cap of the reactor was not fully tightened for aeration. Reactors were shaken at 200 revolutions per minute (rpm) for desired reaction time (~ 6 h). After reaction, the bulk phase (liquid phase) of each

reactor was separated from AIM media (solid phase) using a magnet and collected into a fresh sterile bottle. Collected samples were serially diluted with normal saline solution and the live *E. coli* concentration was measured.

The remaining AIM solids were used to evaluate the concentration of adsorbed bacteria. The solid media was washed three times with normal saline solution, then filled with the same volume (10 mL) of fresh normal saline. Each reactor was applied sonication (Bransonic 220, USA) for 30 minutes to desorb the attached *E. coli.* The liquid in the sonicated reactors was then collected and used to evaluate desorbed *E. coli*. In order to avoid any possible interference and osmotic stress to the bacteria, all experiments were performed in normal saline solution. The bacteria reduction experiments were all performed in duplicate and repeated at least three times.

III.2.5 ROS measurement

Experimental design for ROS measurement was the same method as ROS scavenger assay except for addition of each reagent for measuring hydroxyl radical, superoxide anion, and singlet oxygen, instead of bacterial suspension. Liquid (bulk phase) samples were collected after 6 h of reaction and used for ROS measurement. All the reagents for measuring ROS were freshly prepared before experiments.

Aqueous hydroxyl radical can be trapped by sodium benzoate (BA) and make a complex called 4-hydroxybenzoic acid (*p*-HBA) (Cheng et al. 2016). Therefore, hydroxy radical was measured indirectly by analyzing *p*-HBA. *p*-HBA was measured by high pressure liquid chromatography (HPLC, Agilent) equipped with a UV detector and C-18 column (4.6 x 250 mm). The mobile phase was a mixture of 0.1% trifluoroacetic acid aqueous solution and acetonitrile (65:35, v/v) at a flow rate of 1 mL/min, with the detection wavelength at 255 nm.

Superoxide radical ($O_2^{-\cdot}$) production was quantified by 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction method (Erdim et al. 2015). The reduction of XTT by $O_2^{-\cdot}$ results in the formation of XTT-formazan, which has a unique absorbance at 470 nm and the formazan was measured by UV–vis spectrophotometer.

Singlet oxygen concentration was measured by using furfuryl alcohol (FFA) as an indicator (k (FFA + $^1O_2$) = 1.2 x 10^8 $M^{-1}S^{-1}$) (Erdim et al. 2015) and measured by gas chromatography-mass spectrometry (GC-MS, Shimadzu) equipped with capillary column (SH-Rxi-tsil MS, 30 m x 0.25 mm x 0.25 µm, Shimadzu). The injector was operated in the split mode at a ratio of 1:50, and helium was used as carrier gas, and flow rate of the gas was 1 mL/min. To prepare samples for FFA analysis, 5 mL of ethyl acetate was added to 5 ml aliquot of the collected bulk phase sample for liquid-liquid extraction. Extraction time was determined to 20 minutes with shaking at 200 rpm since there was no increase in FFA concentration when longer than 20 min of extraction was applied (data not shown). After extraction, 1 ml of ethyl acetate layer was collected to GC vial and analyzed. In order to maintain the loss of FFA in first-order, it is necessary to limit initial [FFA] < 30 ppm (Erdim et al. 2015). In addition, we confirmed that there was a linear relationship between initial FFA concentration in water and FFA concentration in ethyl acetate after liquid-liquid extraction only when the FFA concentration was not higher than 30 ppm. Therefore, 20 ppm of initial FFA was selected as optimum concentration for this study. In addition, the reaction of $^1O_2$ and FFA is known to be independent of pH between 5-12 (Hou and Jafvert 2009).

III.2.6 pH, Fe^{2+} and total Fe (Fe^T) measurement

After reaction, part of suspension (bulk phase) was filtered through a 0.25 µm syringe filter for Fe^{2+} and Fe^T measurement. The residual suspension was used for pH measurement.

Aqueous Fe^{2+} and Fe^{T} were measured by 1,10-phenanthroline method. The partial filtrate was collected in a colorimetric tube and acidified using 0.1 mL 6.0 M HCl for the analysis. pH, Fe^{2+}, and Fe^{T} were analyzed instantly for all samples to avoid further oxidation or interference.

III.2.7 Statistical analysis

Experiments were performed at least three times throughout the study and all data are presented as the mean ± sample standard deviation of repeated values. Statistical significance was determined using Student's t-tests by evaluation of differences by comparing to control or blank groups and analysis of variance (ANOVA) test; $*p < 0.05$ or $**p < 0.01$ were considered statistically significant based on the tests. JMP pro 13 was used as a software tool for statistical analysis.

III.3 Results

III.3.1 Effects of AIM and ROS scavengers on *E. coli* removal

In the previous study, we demonstrated the effect of AIM against *E. coli* removal (Han et al. 2019). To better understand the inactivation mechanism of AIM, ROS scavenger assay was conducted in this study. The initial concentration of each scavenger was 0.28 M, 5 mM, and 100 μM for DMSO, SA, and MnTBAP, respectively, and 3 h of reaction time was given (Han et al. 2019). Fig. 10 (a) shows that AIM removed significant amount of bacteria (> 4 log) in bulk phase without scavengers (control). In addition, addition of DMSO and MnTBAP, which are scavenger of hydroxyl radical and superoxide anion, respectively, have negligible effects on bacterial removal by AIM. However, SA significantly inhibited the bacterial removal efficiency of AIM in bulk phase (Fig. 10 (a)).

Sonication (30 minutes) was applied to residual AIM solid phase after reaction in order to analyze adsorbed live *E. coli*. No effect was observed on *E. coli* viability by sonication itself within 30 min (Han et al. 2019). Fig. 10 (b) shows that *E. coli* was not detected in solid phase after sonication in DMSO or MnTBAP added group, and negligible live cells (< 10 CFU/ml) were observed in control. However, it was observed that SA inhibited the bactericidal activity of AIM in the solid phase (Fig. 10 (b)). In addition, concentration of SA also affected the AIM performance. Fig. 11 shows that the inhibition of bacterial removal was increased with the concentration of SA added into the system in both solid and liquid phase. These results suggested that there is a high possibility of singlet oxygen generation during oxic reaction of AIM, and the singlet oxygen generated from AIM could be responsible for bacterial inactivation.

III.3.2 Effects of reaction time with SA on singlet oxygen generation

Fig. 12 indicates the effect of reaction time of AIM depending on availability of SA on *E. coli* removal in a bulk phase. At the initial phase of the reaction (less than 1 min), significant amount of *E. coli* (2.5-2.8 log) was removed by adsorption (Han et al. 2019). After the initial reduction, the *E. coli* concentration did not significantly decrease in the SA treated group until 360 min of reaction.

(a) Bulk phase

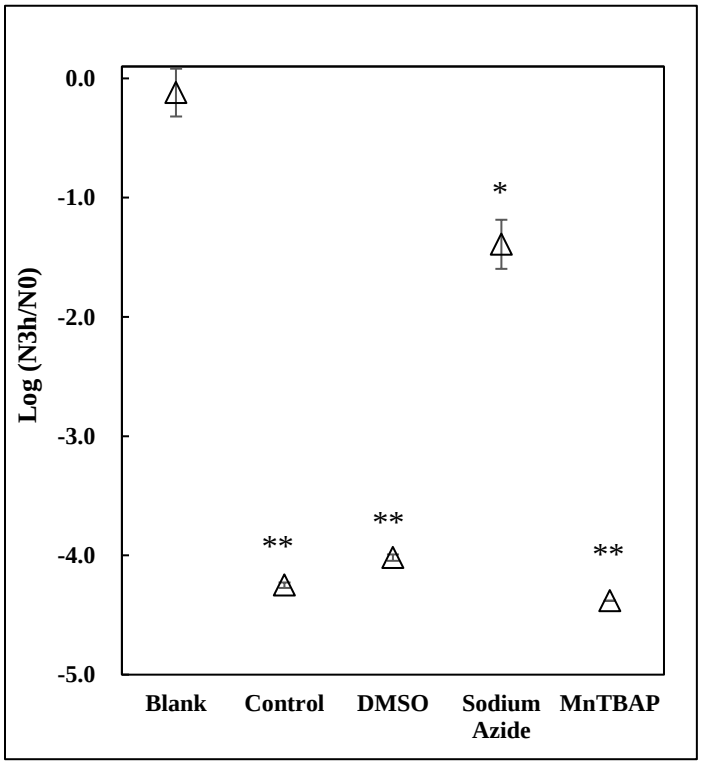


Figure 10. Effect of ROS scavengers on bacterial removal. (a): Bulk phase and (b): Solid phase. Statistical significance was determined by evaluation of differences by comparing to (a) blank or (b) control; *$p < 0.05$ or **$p < 0.01$ were considered statistically significant based on the tests.

(b) Solid phase

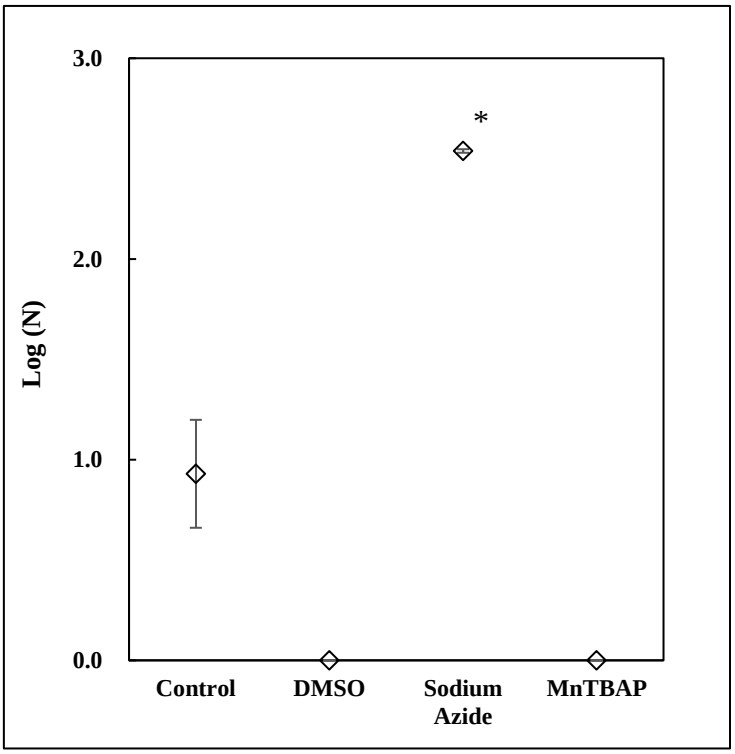
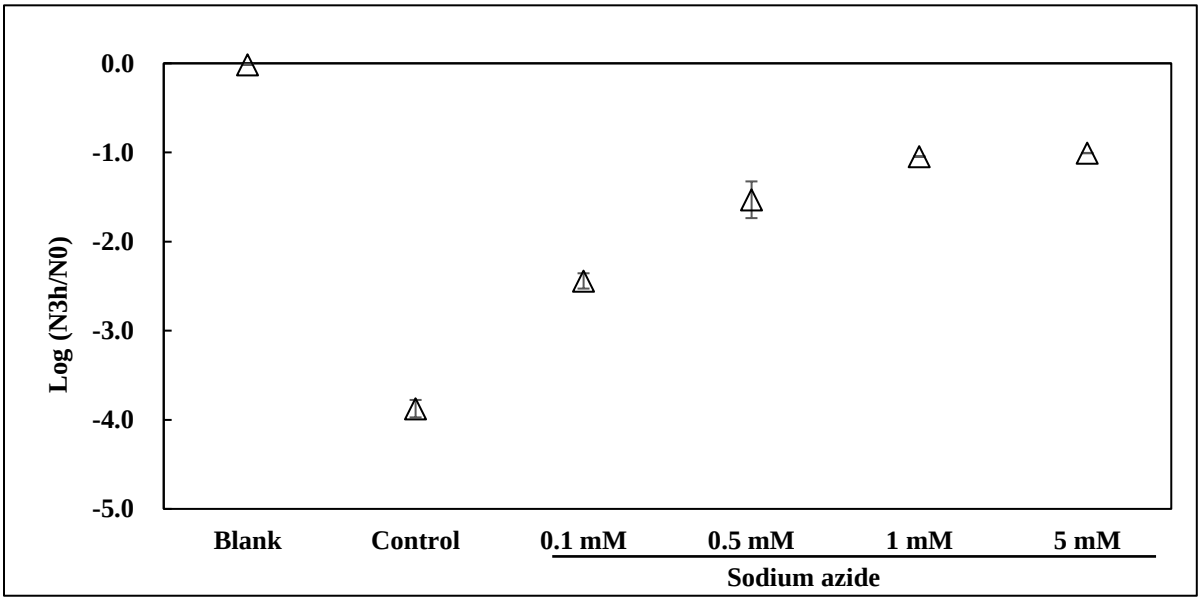


Figure 10. Continued.

(a) Liquid phase



(b) Solid phase

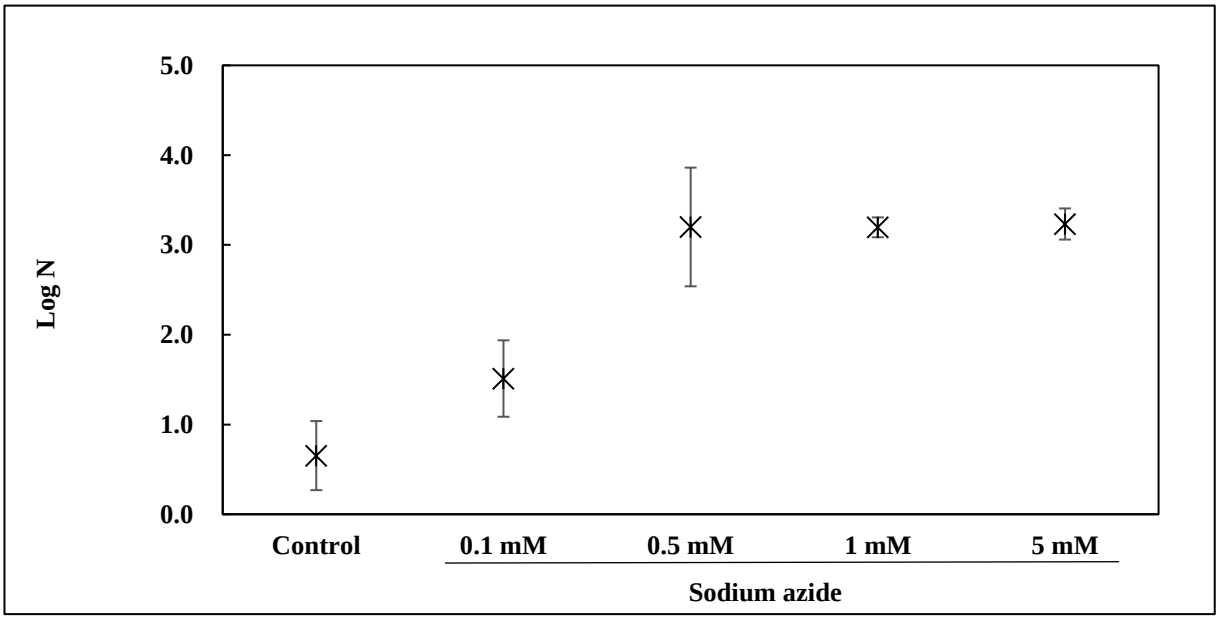


Figure 11. Effect of sodium azide concentration on *E. coli* removal by AIM; (a) liquid phase and (b) solid phase.

There was up to 2.04 log scale different live *E. coli* cells in bulk phase between with and without SA addition, and this difference increased with reaction time. Addition of 0.1 mM Fe^{2+} with 5 mM SA (control) did not show a significant effect (less than 1 log scale) on *E. coli* viability during 6 h (Fig. 12). We could not add more than 5 mM of SA because greater than 5 mM of SA has a toxicity on *E. coli* (data not shown). Therefore, 5 mM of SA was chosen for the further experiments in this study.

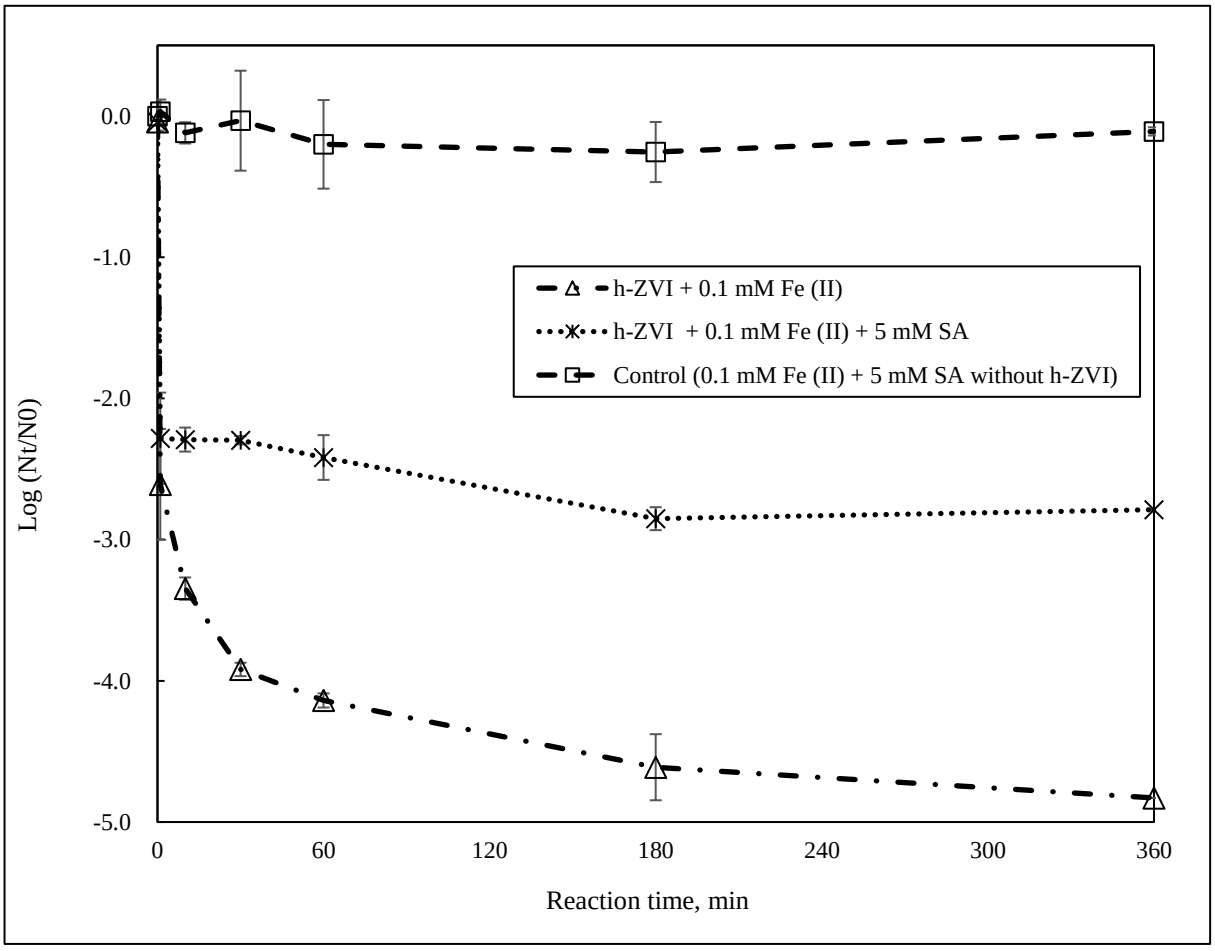


Figure 12. Effect of reaction time of AIM and singlet oxygen scavenger on *E. coli* viability.

III.3.3 ROS generation by ZVI, Fe^{2+}, and AIM media

Fig. 13 (a) indicates singlet oxygen generation by reaction of ZVI, Fe^{2+}, and AIM within 6 h in the presence of oxygen. Singlet oxygen concentration was determined indirectly by FFA concentration change and represented as C_t/C_0 ($[FFA]_t$ / $[FFA]_{initial}$). The FFA concentration was significantly reduced (C_{6h}/C_0 = 0.66) when 3 mM of Fe^{2+} was externally added with AIM. However, there was no decrease in FFA concentration from pure ZVI. Fe^{2+} itself showed slight reduction of the FFA (C_{6h}/C_0 = 0.94), and this change was statistically significant in 95% confidence level (*p*-value = 2.1%) compared to blank. When singlet oxygen scavenger (5 mM SA) was added, the FFA concentration change was significantly inhibited (C_{6h}/C_0 = 0.87). The effect of reaction time on singlet oxygen generation is shown in Fig. 13 (b). The concentration of externally added Fe^{2+} was 1.5 mM since the result in Fig. 14 (a) showed that there was no increase in singlet oxygen generation when greater than 1.5 mM Fe^{2+} was added. The time course result shows that the singlet oxygen was gradually generated with reaction time in 6 h duration (Fig. 13 (b)).

Effect of externally added Fe^{2+} concentration was also investigated. 0 – 3.0 mM of Fe^{2+} was added and reacted with AIM media for 6 h. The results showed that the AIM reduced FFA without additional Fe^{2+} (C_{6h}/C_0 = 0.87) and the concentration of FFA decreased with increase of Fe^{2+} concentration up to 1.5 mM (Fig. 14 (a)). There was no significant difference in FFA concentration when greater than 1.5 mM of Fe^{2+} was added. When ZVI was applied without preconditioning, there was no reduction in FFA concentration for 6 h duration (95% confidence level ANOVA test, *p*-value = 45.3%), even in the highest dosage of Fe^{2+} in the experimental condition (Fig. 14 (b)).

Effect of AIM on ROS generation besides singlet oxygen was investigated because previous studies reported superoxide anion and hydroxyl radical can be generated by ZVI nano particle (NP) (Kim et al. 2010). However, any evidence for these ROS (superoxide anion and hydroxyl radical) generation was not found with AIM in the experimental condition (data not shown).

(a) Comparison of singlet oxygen generation by different iron media

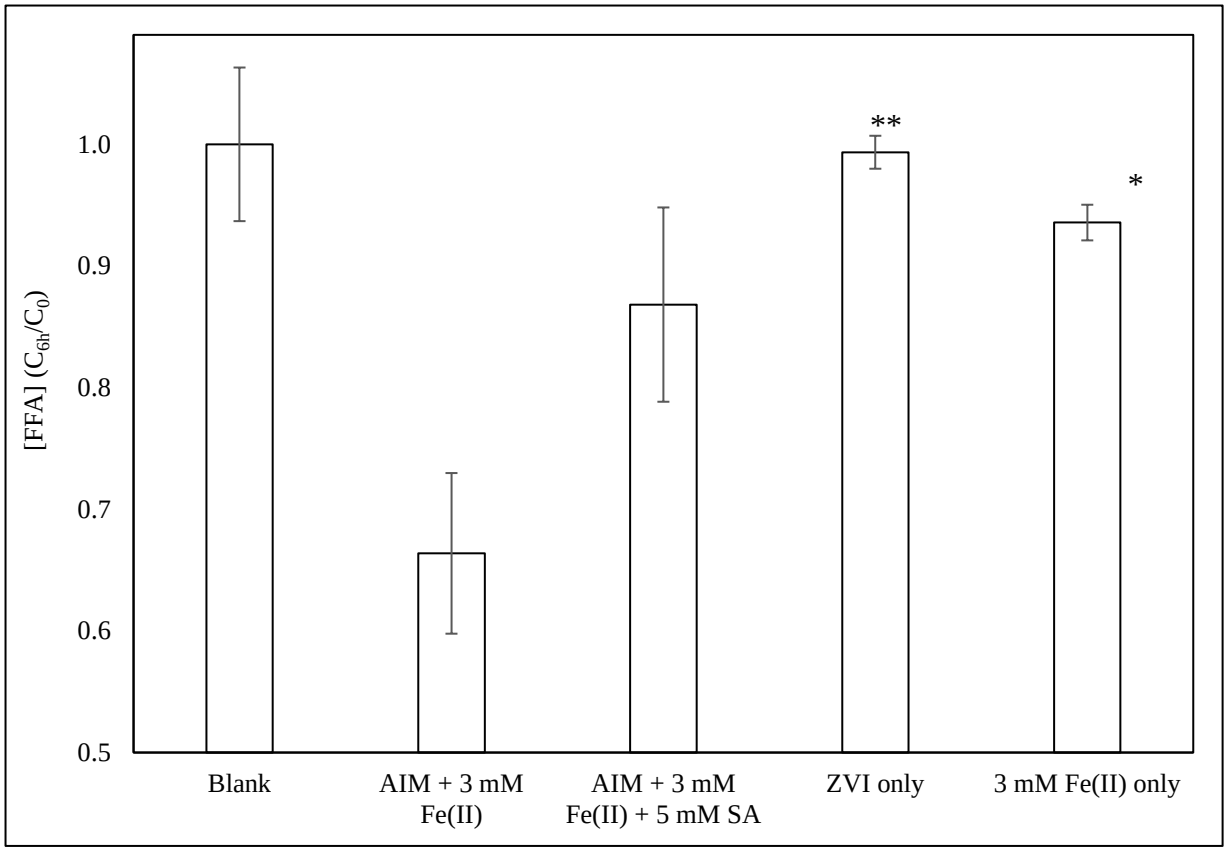


Figure 13. (a) Comparison of singlet oxygen generation by different iron media and (b) effect of reaction time on singlet oxygen generation by AIM media. Statistical significance was determined by evaluation of differences by comparing to (a) blank or (b) initial (0 h); *$p < 0.05$ or **$p < 0.01$ were considered statistically significant based on the tests.

(b) Singlet oxygen generation by AIM in time dependent manner

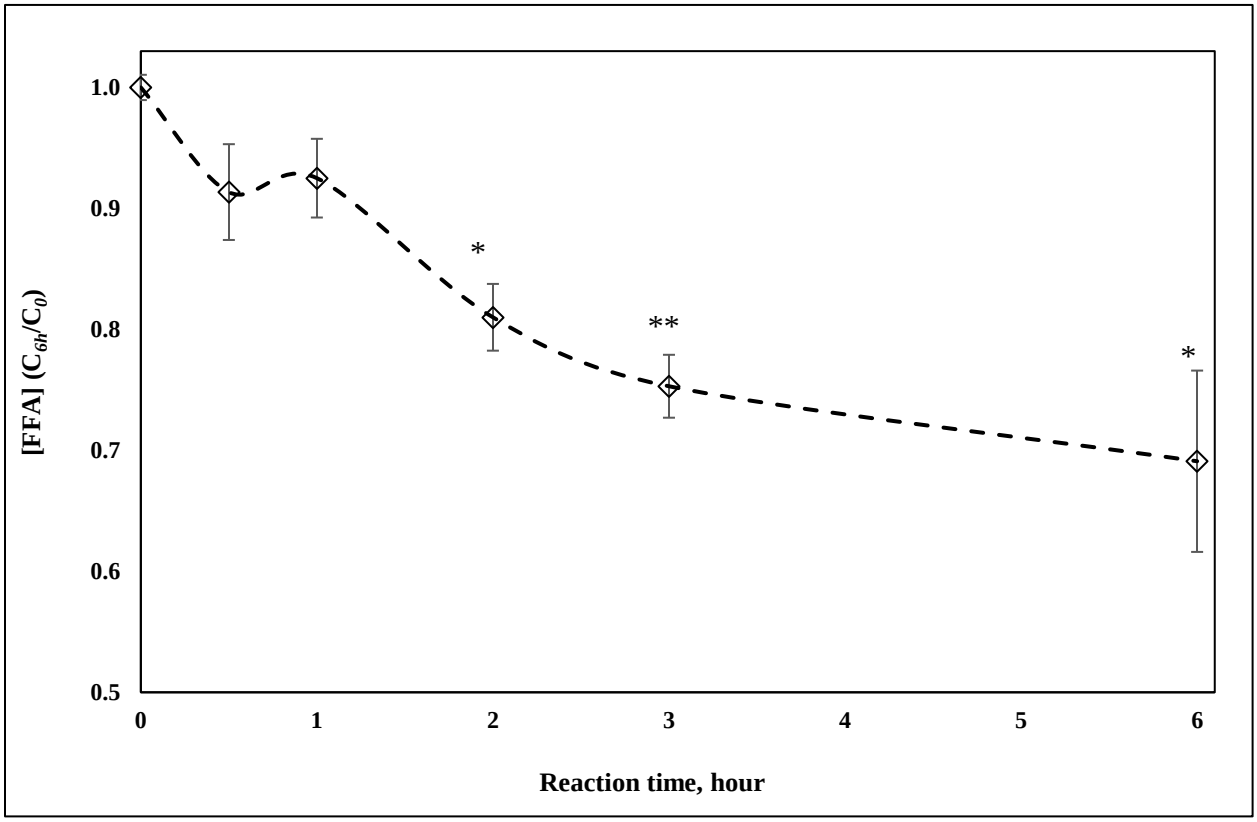
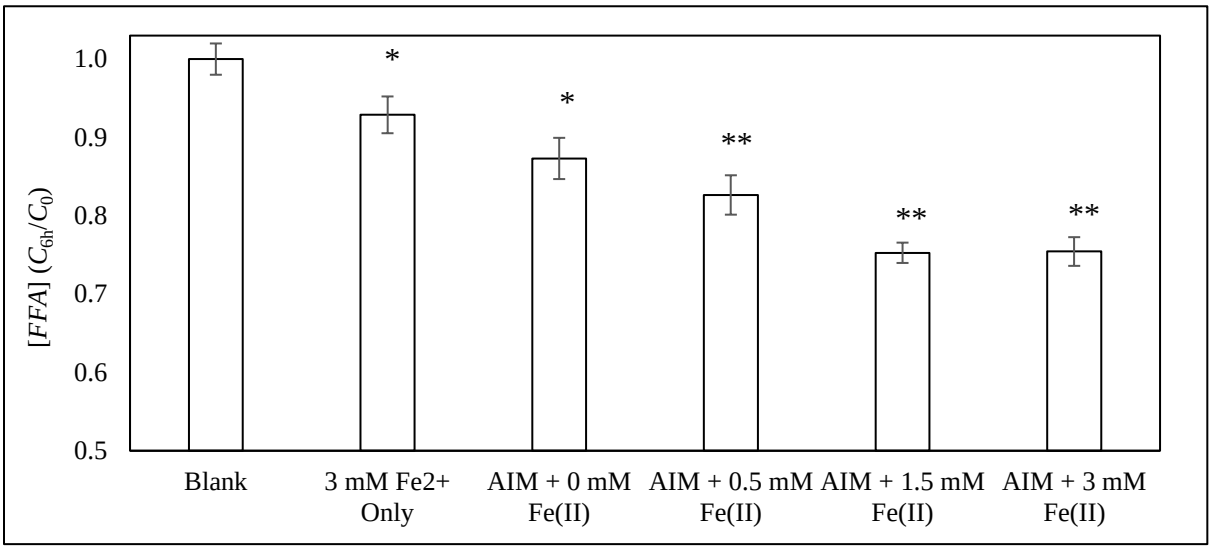


Figure 13. Continued.

(a) AIM



(b) ZVI

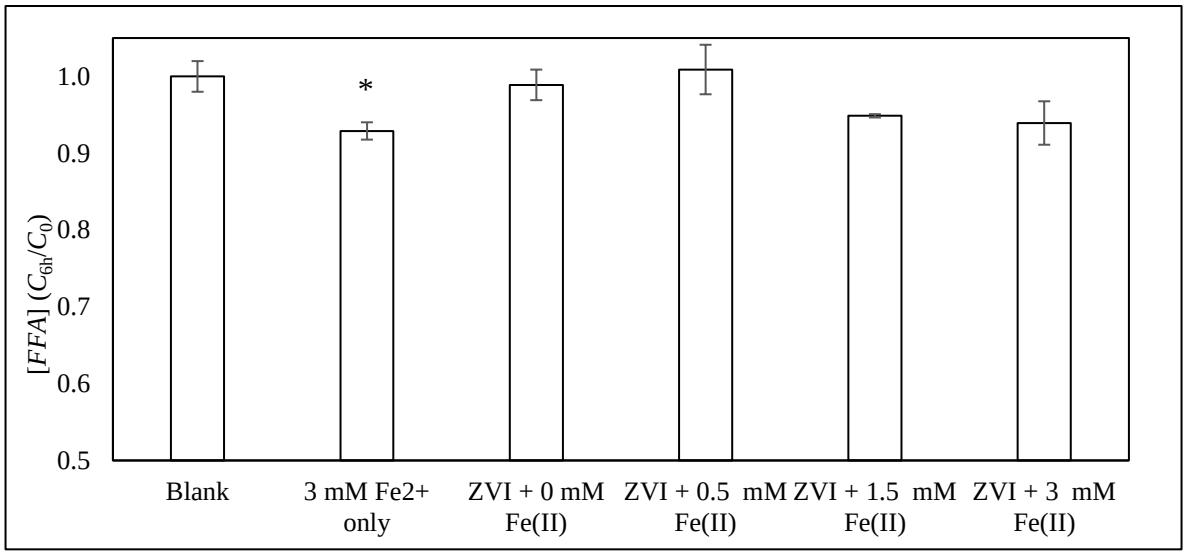


Figure 14. Effect of externally added Fe (II) concentration on singlet oxygen generation. (a): AIM, (b): ZVI without preconditioning. Statistical significance was determined by evaluation of differences by comparing to blank; $*p < 0.05$ or $**p < 0.01$ were considered statistically significant based on the tests.

III.4 Discussion

Previous studies reported that magnetite was the dominant corrosion product as a result of iron-nitrate redox reaction (Huang et al. 2003; Huang et al. 2005). Once magnetite coated on the source iron grains, negligible nitrate reduction occurred if the solution contained a limited amount of Fe^{2+}. In the experimental system in our study, nitrate played a role as an electron acceptor and assumed to be completely reduced to ammonia during preconditioning process. In addition, externally added Fe^{2+} helps to form of a black oxide coating (magnetite) (Huang et al. 2003; Huang et al. 2005). As long as Fe^{2+} is available in the aqueous phase, magnetite could be formed and coated onto the surface of ZVI (Huang et al. 2013; Huang et al. 2012; Tang et al. 2016). The reaction during the ZVI preconditioning can be summarized as following (Han et al. 2019; Huang et al. 2005):

$$NO_3^- + 2.82\ Fe^0 + 0.75\ Fe^{2+} + 2.25\ H_2O \rightarrow NH_4^+ + 1.19\ Fe_3O_4 + 0.50\ OH^-$$

The major finding and advantage of creating AIM through preconditioning method is producing a magnetite coating on the ZVI surface, which allows more favorable reaction status of iron oxide (Huang et al. 2005). The primary role of Fe^{2+} in the system was to facilitate ferric (hydr) oxides, the dominant passive corrosion products, transformation to magnetite (Huang et al. 2013; Huang et al. 2012; Tang et al. 2016). In this manner, ZVI preconditioning could overcome ZVI surface passivation and keep reactivity of the core ZVI. In terms of bacterial removal efficiency, magnetite itself has limited bactericidal activity but has great adsorption capacity (Lee et al. 2008). These characteristics of magnetite allowed the AIM to effectively remove and inactivate bacteria by simultaneous adsorption and redox reaction (Han et al. 2019). The purpose of this study was to evaluate the mechanism of bacterial inactivation of AIM, focused on ROS generation during AIM oxic reaction.

According to studies have been reported, main ROS products caused by ZVI (or ZVI NP) oxic reaction are superoxide anion, hydroxyl radical, or ferryl radical (Wu et al. 2014; Kim et al. 2010). These radicals are known as the major factors affecting bacterial viability. For this reason, ROS scavenger assay was performed in this study in order to elucidate whether a specific ROS generated from AIM is responsible for bactericidal inactivation. As shown in Fig. 10, 5 mM of SA, the singlet oxygen scavenger, significantly inhibited bacterial removal by AIM. Superoxide anion and hydroxyl radical scavenger did not reduce the bacterial removal efficiency. Since SA is known as a specific singlet oxygen quencher (Li et al. 2001; Bancirova 2011), this inhibition can be interpreted that SA quenched singlet oxygen generated from AIM reaction. In another word, it could be concluded that singlet oxygen is the main reason of bacterial inactivation in the AIM system.

Kim et al. demonstrated that ROS generated from ZVI NP was not directly related to bacterial viability in the bulk phase (Kim et al. 2010). The authors of this study suggested that a limitation on a diffusion of ROS scavengers to the interface of ZVI and bacteria particles could be a possible reason. Because of this reason, it was difficult to show the effect of scavengers in a short period of experimental condition. To minimize this diffusion limitation issue and confirm the effect of scavenger in the bulk phase more accurately, we added each scavenger (DMSO and MnTBAP) from the beginning of bacterial culture stage. DMSO and MnTBAP were added to TSB at the same concentration as the experimental condition, and repeated the scavenger assay with the bacteria came from scavenger included media. We assumed that this experimental design could provide enough time to overcome the diffusion limitation. However, any scavenger effect was not observed even in this experimental condition (Fig. 15). This result demonstrates

that superoxide anion and hydroxyl radical were not generated and/or directly related to the bactericidal effects in AIM system.

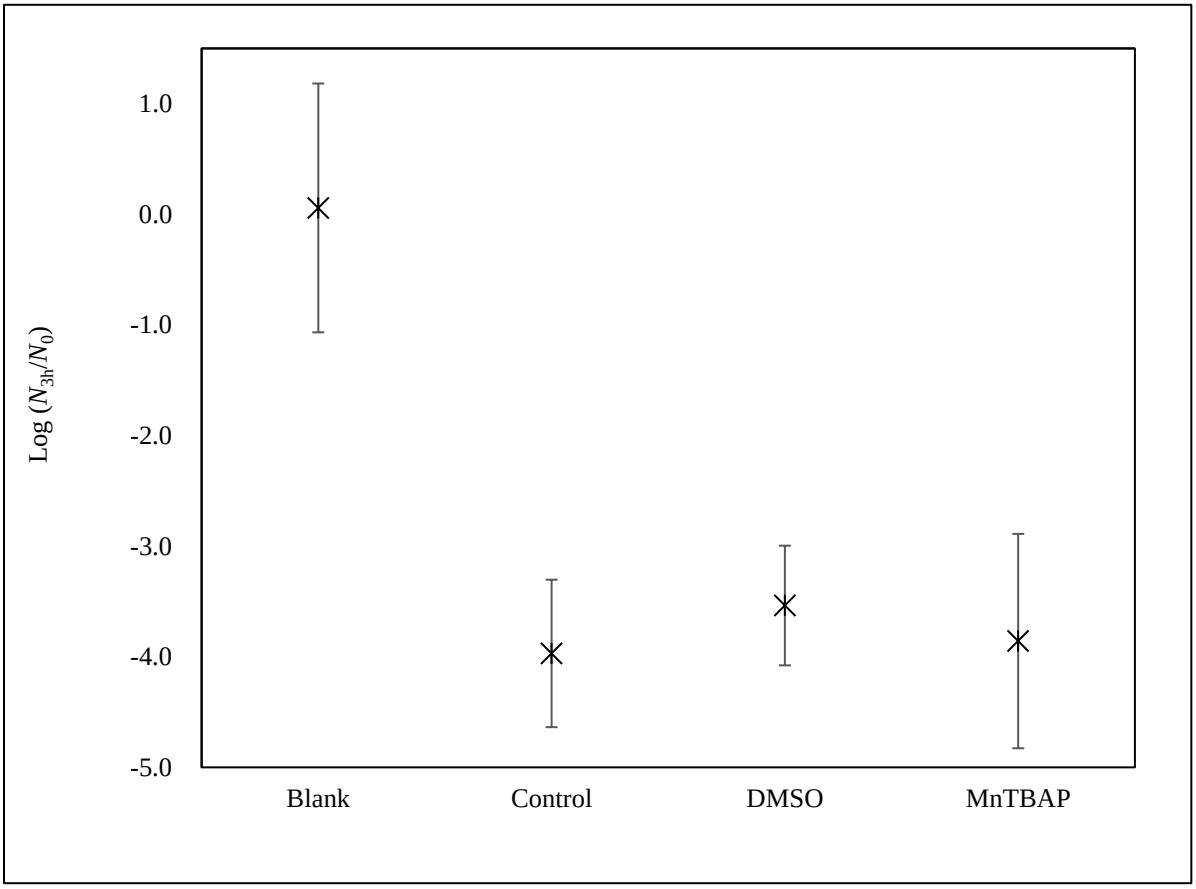


Figure 15. Effect of scavengers for hydroxyl radical and superoxide anion on bacterial removal. Each scavenger was added into the media from the bacterial culture suspension in order to prevent a diffusion issue of the scavengers.

In this study, because relatively high concentration of sodium (SA) was added as a scavenger, it could increase an ionic strength and result in decrease of bacterial adsorption. Thus, zeta potential of *E. coli* particle after reaction was measured in order to verify whether the effect of SA on bacterial reduction is caused by singlet oxygen quenching or reduced adsorption capacity.

Fig 16 shows the zeta potential differences of bacteria particle in different reaction conditions. There was no significant difference between two groups, “AIM + Fe (II)” and “AIM + Fe (II) + SA” (*p*-value = 11.6%), which supports that there is negligible effect on adsorptive removal of bacteria caused by addition of SA. Therefore, we could conclude that the major factor of bactericidal effect of AIM is singlet oxygen. As shown in in Fig. 12, there was a significant

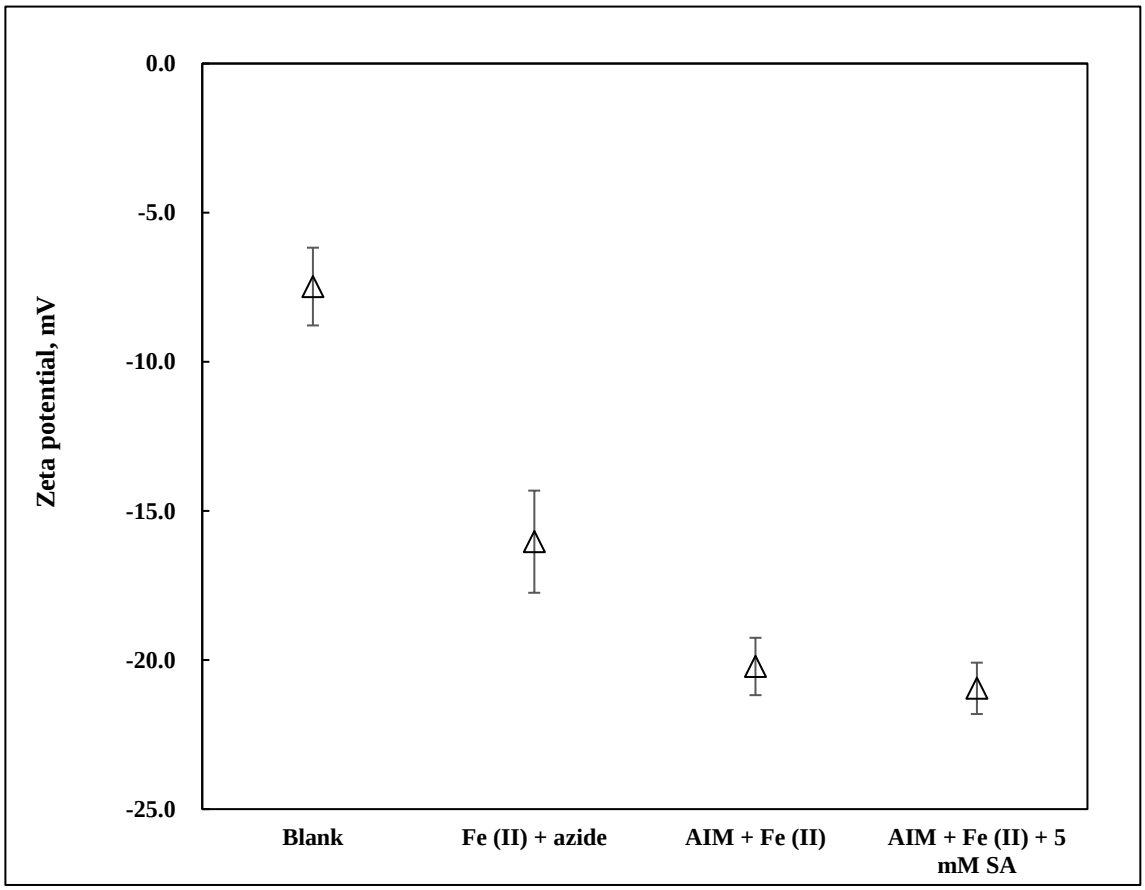


Figure 16. Effect of sodium azide on zeta potential of bacterial cell particles.

bacterial reduction (~ 2.2 log) within 1 min of reaction in both of control and scavenger treatment then, bacterial reduction rate decreased substantially in the scavenger treatment group. This result also supports that SA did not affect bacterial adsorption. In addition, we could confirm that bacterial adsorption by AIM is very rapid process followed by inactivation. When 5 mM of SA was added, the bacterial concentration slightly decreased after 6 h reaction compared to initial reaction phase (Fig. 12), and FFA concentration after 6 h in the scavenger group is also slightly lower than blank or Fe^{2+} only group (Fig. 13 (a)). These results indicate that 5 mM of SA was gradually consumed by singlet oxygen during the reaction, resulted in losing singlet oxygen quenching ability at longer reaction time. This could be another evidence that singlet oxygen is the major factor of bacterial inactivation in AIM system. Experiments with higher dosage of SA could confirm this evidence, but we could not add greater than 5 mM of SA because of the toxicity of SA itself against *E. coli* in higher concentration.

On the contrary to the result of AIM, there was no evidence of singlet oxygen generation with oxic reaction without preconditioning (Fig. 13 (a) and 14 (b)) even in the highest concentration of additional Fe^{2+}. These findings suggest that magnetite and externally added Fe^{2+} have a crucial role in singlet oxygen generation. ZVI oxidation has been proved to produce ROS under ambient condition, however, this metabolism is substantially limited by other competing reactions, which results in loss of reactive iron species (ferrous and ferric ions) in the form of iron corrosion products, such as iron oxides and hydroxides (Bancirova 2011; Joo et al 2005). These competing reactions in oxygenated systems rapidly reduce the efficiency of the n-ZVI through passivation of ZVI surfaces by corrosion products, result in decrease of the electron transfer rate. Recently, it has been reported that in ZVI systems the efficiency of electron transfer processes and the yield of ROS production could be enhanced using ligands (e.g.,

ethylenediaminetetraacetate (EDTA)), electron shuttles (e.g., polyoxometalate; a metal–oxygen anion), or natural organic matter (Davenport et al. 2000; Keenan and Sedlak 2008; Lee et al. 2008). Thus, results of this study indicate it is possible that magnetite can be act as an electron shuttle during ZVI oxidation.

Fig. 17 indicates the result of singlet oxygen generation by oxic reaction of magnetite (commercially purchased) with additional Fe^{2+}. Because the purchased magnetite powder (Alpha chemical) was not stored in anoxic condition, the surface can be oxidized and lose its reactivity. Thus, magnetite was activated with deoxidized DI water and 7 mM of Fe^{2+} before use. Magnetite surface activation was conducted similar method to the ZVI preconditioning method. Fifteen (15) mL centrifuge tubes with cap were used as the reactor for preparing the activated magnetite. As the first step, 0.5 ± 0.002 g of magnetite was added into the reactors, which was then transferred into the anaerobic chamber. 7 mM of Fe^{2+} and DDI water were pipetted into the reactor to achieve a reactant solution of 10 mL in total. The reactors were sealed with cap in the anaerobic chamber and then transferred into a rotary tumbler for complete mixing at 30 rpm at 25 ± 2°C for 48 h in the dark. As shown in Fig. 17, there were negligible change in FFA concentration with activated magnetite regardless of Fe^{2+} concentration. It is known that magnetite is the end product of iron oxides in the experimental condition and stable (Huang and Zhang 2005), as well as there is no other electron donor (i.e. ZVI), there should be not enough redox energy for producing singlet oxygen by magnetite itself.

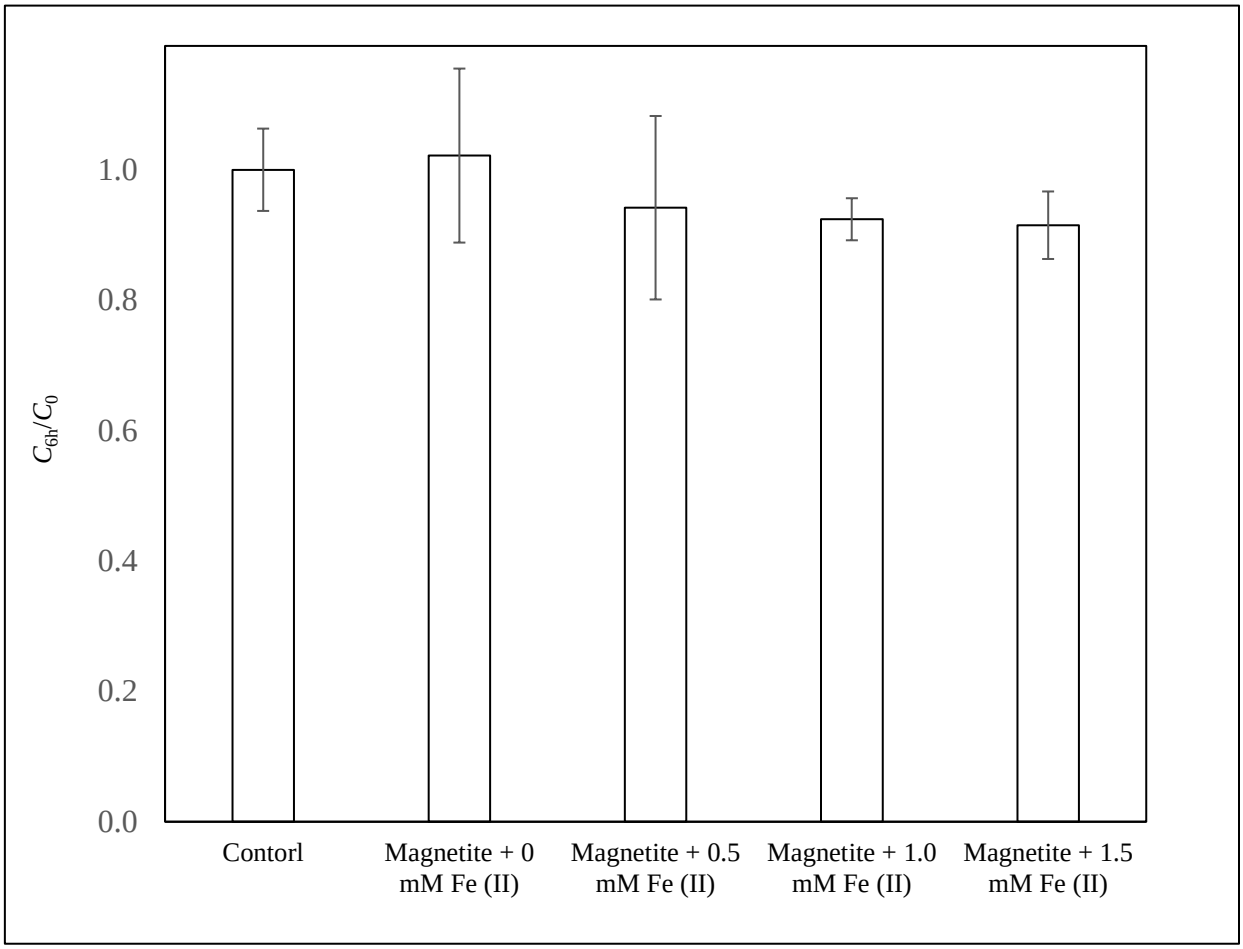


Figure 17. Effect of activated magnetite on singlet oxygen generation. The singlet oxygen was analyzed by measuring FFA and the result was indicated as C_{6h}/C_0 ($[FFA]_{6h}$ / $[FFA]_{initial}$).

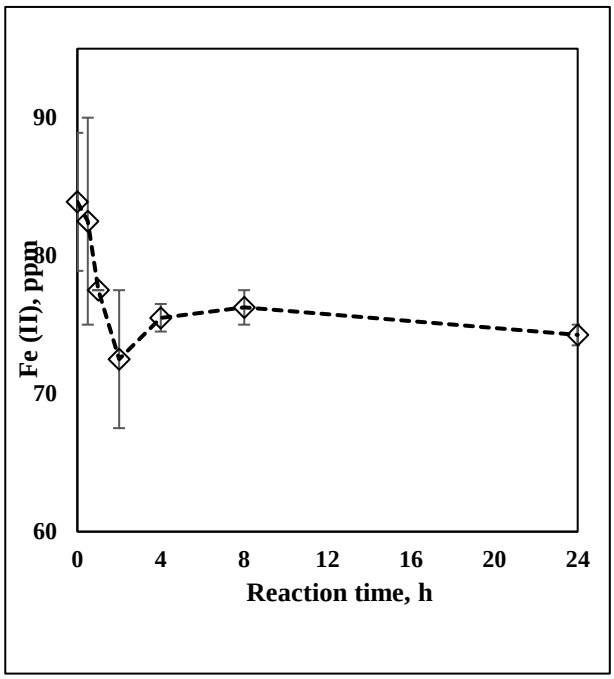
Singlet oxygen refers to singlet electronic excited states and the singlet states of oxygen are higher in energy than the triplet ground state of oxygen. Typically, it is known that ground state oxygen can be transited to excited energy state by external photoenergy such as ultraviolet and/or visible light (Kang and Choi 2009). Other studies found that chemical energy (i.e. chemiluminescence) is also able to generate singlet state oxygen (Fu et al. 2014). Energy

required to elevate oxygen to singlet states is 1270 nm (= 0.976 eV), and $Fe^0 \rightarrow Fe^{3+}$ oxidation can generate greater than 0.976 eV in neutral pH (Koppenol 1976; Saito and Nosaka 2014). Therefore, singlet oxygen generation by redox reaction of ZVI is thermodynamically allowed process and it is possible that redox reaction of AIM could be closely related to singlet oxygen generation.

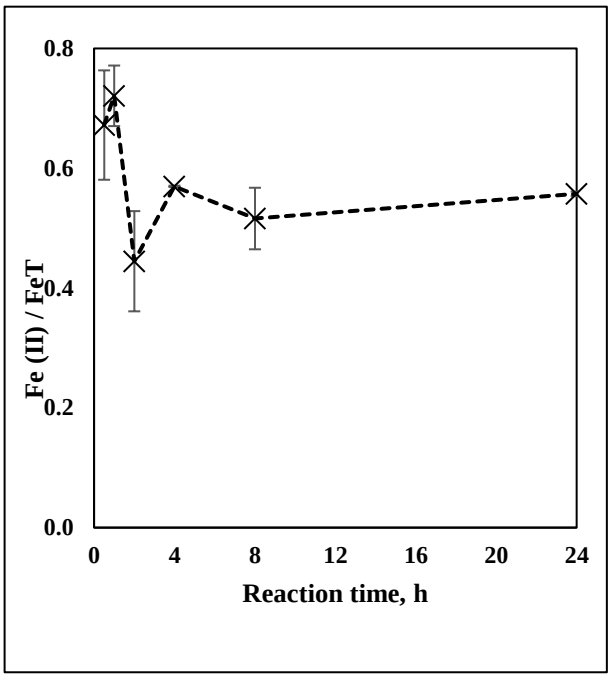
Aqueous Fe^{2+} concentration and Fe^{2+}/Fe^T ratio were measured after AIM oxic reaction. Fig. 18 (a) and (b) show the change of aqueous Fe^{2+} concentration and Fe^{2+}/Fe^T ratio by reaction time. The aqueous Fe^{2+} concentration slightly decreased at the beginning stage (~ 10 ppm) and kept stable until end of reaction (24 h). This result corresponds to the previous result (Huang and Zhang 2005) and represents that major electron donor of the AIM reaction is ZVI, not aqueous Fe^{2+}.

Comparing the Fe^{2+}/Fe^T ratio change caused by reaction of AIM, activated magnetite, and ZVI (Fig. 18 (b), Fig. 19 (a), and (b), respectively), the aqueous Fe^{2+}/Fe^T ratio of AIM after 24 h was significantly lower those that of activated magnetite and ZVI. Since the aqueous Fe^{2+} did not directly involved in the AIM redox reaction, these results suggest that ZVI oxidized to Fe^{3+} and this oxidation energy is the main source for excitation of ground state oxygen. Therefore, we could conclude that the magnetite was not involved in redox reaction directly, magnetite coating helps to keep a reactivity of ZVI and transfer electrons from ZVI, result in giving higher redox power in the system. Because of the fact that AIM could be acted as buffer (Tang et al. 2016), we were able to eliminate the other pH effect on iron redox reaction in the system. Indeed, pH of solution was stable in near neutral during the reaction (Fig. 18 (c))

(a)



(b)



(c)

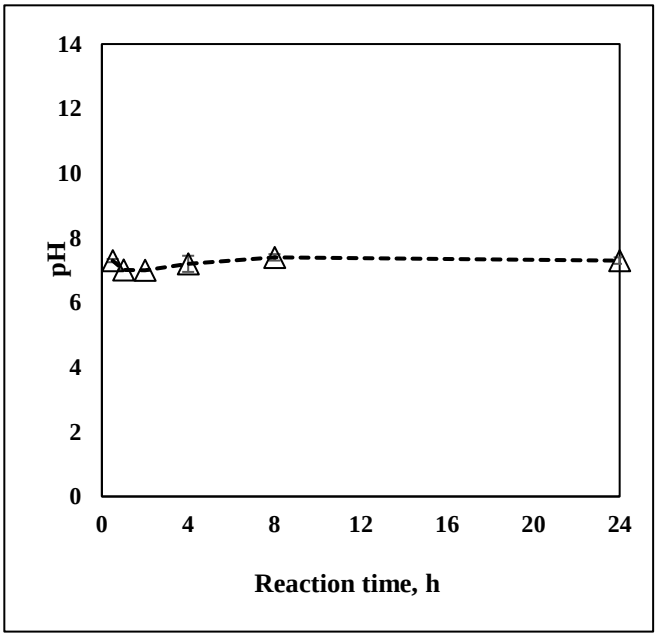
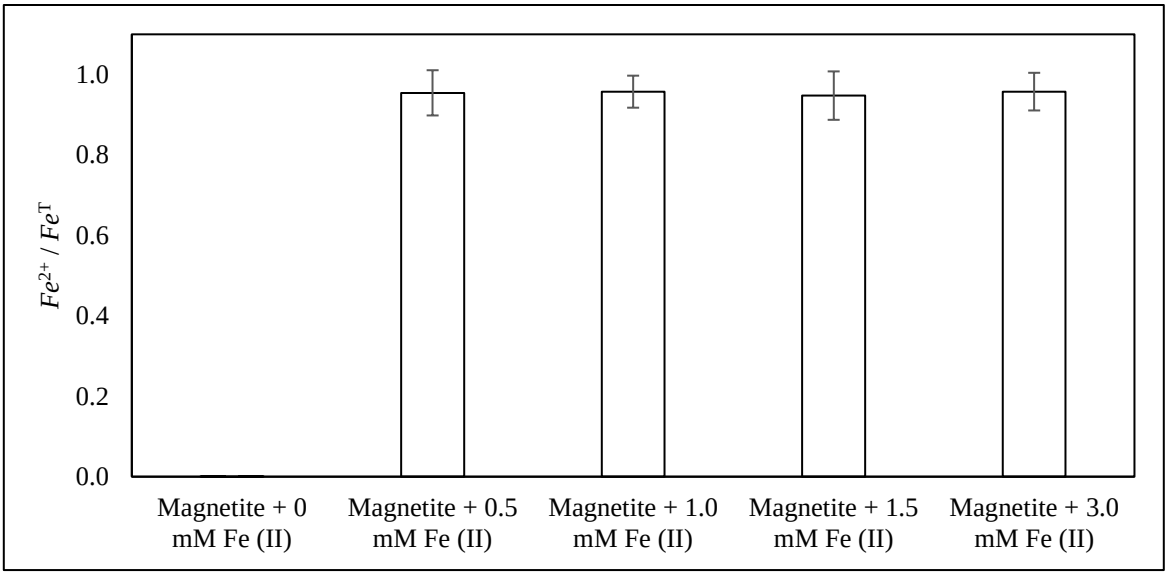


Figure 18. (a) Aqueous Fe (II) concentration; (b) Fe (II) / FeT ratio, and (c) pH change by AIM oxic reaction with 1.5 mM Fe (II).

(a)



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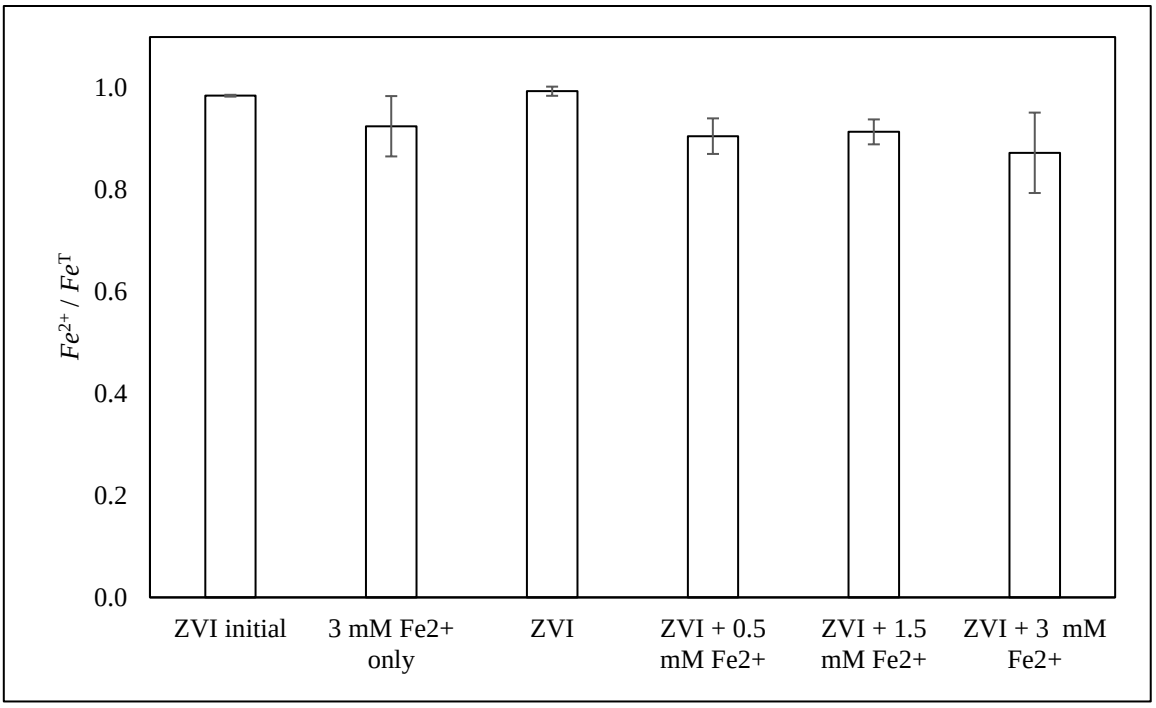


Figure 19. Aqueous Fe^{2+}/Fe^{T} ratio after 24 h oxic reaction of (a) activated magnetite and (b) ZVI.

In terms of thermodynamic energy, ZVI to Fe^{3+} oxidation process might be a possible factor for singlet oxygen generation. Main iron oxide products of ZVI oxidation in standard condition is lepidocrocite (γ-FeO(OH)) and maghemite (γ-Fe_2O_3) (Huang and Zhang 2005). Because AIM is coated by magnetite, which has lower band gap energy, it makes easier electron transfer (Huang and Zhang 2005), which means AIM has higher redox potential compared to regular ZVI. Therefore, singlet oxygen may not be generated or less amount was generated under detection limit in regular ZVI. In addition, considering the fact that hydroxyl radical and superoxide anion were not detected in experimental condition of this study, and the results of FFA concentration change of normal ZVI and AIM (Fig. 4), it can be concluded that singlet oxygen would be a major cause of the bactericidal effect in the AIM system.

III.5 Implication and Further Discussion

In this study, lower Fe^{2+}/Fe^{T} value from AIM reaction compared to the value from regular ZVI strongly suggests that oxidation of ZVI is closely related to singlet oxygen formation. There were several articles related to singlet oxygen generation without external photo energy (Yesilgul et al. 2017; Saito and Nosaka 2014; Corey et al. 1987). One possible mechanism for singlet oxygen formation without light source is superoxide disproportionation. The authors of these study suggest following mechanism (proton assisted singlet oxygen formation) (Saito and Nosaka 2014; Corey et al. 1987);

(1) Superoxide protonation to form $HOO\bullet$

$$O_2^- + H^+ \rightarrow HOO\bullet$$

(2) $HOO\bullet$ reduction by superoxide or;

$$HOO\bullet + O_2^- \rightarrow HO_4 \rightarrow {}^1O_2$$

(3) Disproportionation

$$2\,HOO\bullet + H_2O \rightarrow H_2O_4 \rightarrow H_2O_2 + H_2O + {}^1O_2$$

(4) The overall reaction

$$2\,O_2^- + 2\,H^+ \rightarrow H_2O_2 + {}^1O_2$$

According to the above reaction, hydrogen peroxide and singlet oxygen is generated as the final products in the overall reaction. Accordingly, an additional scavenger assay was performed using 10 mM of sodium pyruvate, a hydrogen peroxide scavenger as shown in Fig. 20. The scavenger assay results in the Fig. 20 indicates that hydrogen peroxide is also involved

in the bacterial removal mechanism of AIM against *E. coli*. However, these results alone do not distinguish whether hydrogen peroxide is directly involved in bacteria removal or indirect mechanism by inhibition of Fenton reaction of AIM in the aqueous system. Prior to the scavenger assay, experiments to evaluate the effects of AIM amount on bacterial removal were conducted in order to exclude other bacteria removal mechanisms such as adsorptive removal, and to maximize the results of bacterial inactivation of ROS.

Fig. 21 shows the time dependent bacterial removal efficiency in different amount of AIM. As a result, the following experiment was conducted using AIM of 0.05 g, which is 10% of the amount used in the previous experiment. Fig. 20 and Fig. 12 show that the initial bacterial removal, which is caused bacterial adsorption, was significantly reduced when a smaller amount of AIM was used. In addition, in order to maintain the initial SA concentration in the SAS group (Sodium Azide Spiked group), a concentrated SA solution was spiked every 1.5 hour as a compensation for the consumption of SA as the reaction time progressed. The results indicate that the bacteria removal efficiency of the SAS group was significantly inhibited compared to the control or SA group. This is a clear and direct evidence showing that SA is consumed according to the reaction time. In addition, since SA is known as a specific quencher of singlet oxygen, this result can be regarded as conclusive evidence that singlet oxygen is a direct cause of bacterial elimination of AIM.

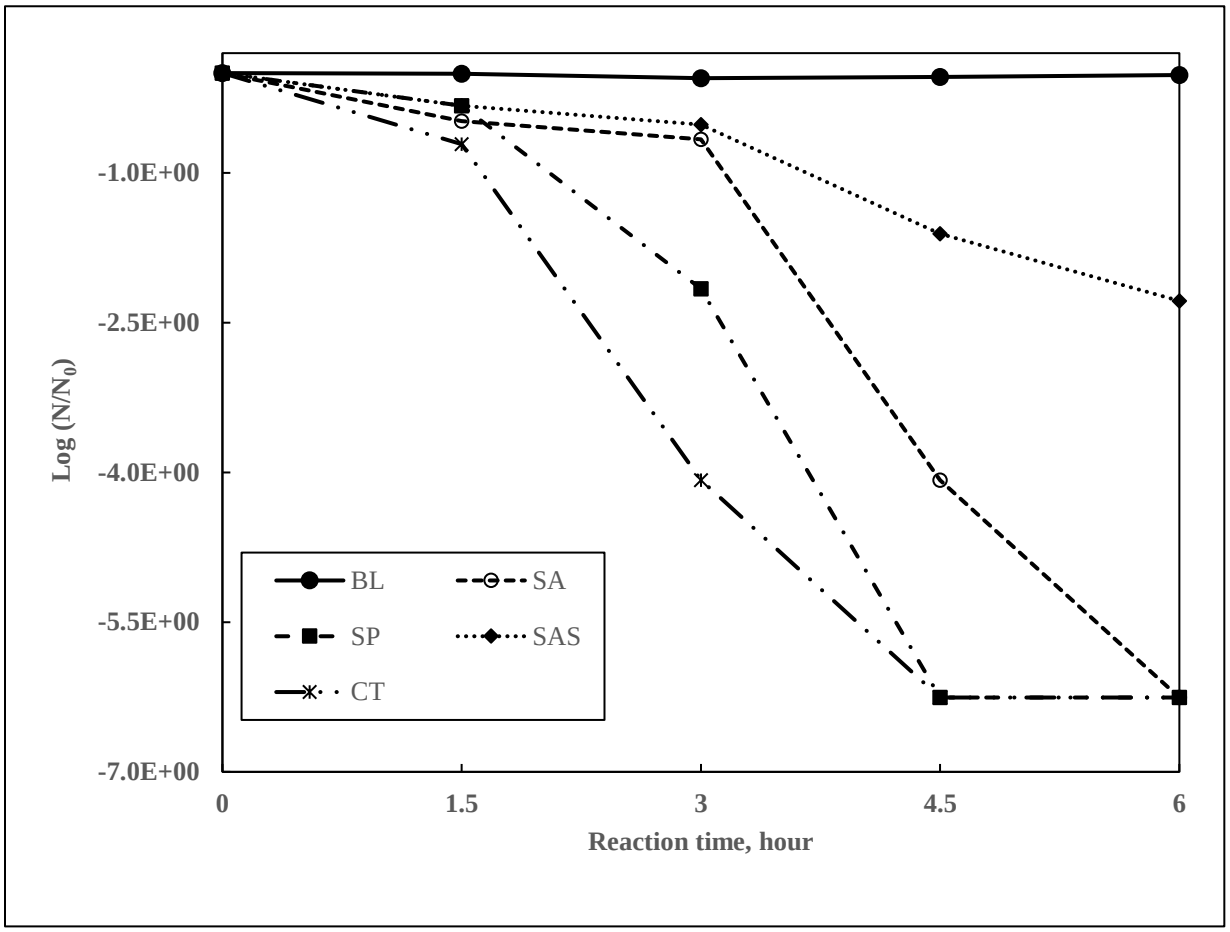


Figure 20. Effect of scavengers for hydrogen peroxide and singlet oxygen on bacterial removal. *BL, Blank (Bacterial only); SP, 10 mM sodium pyruvate; SA, 5 mM sodium azide; SAS, 5 mM sodium aziade spiked every 1.5 hour; CT, control (without scavenger).

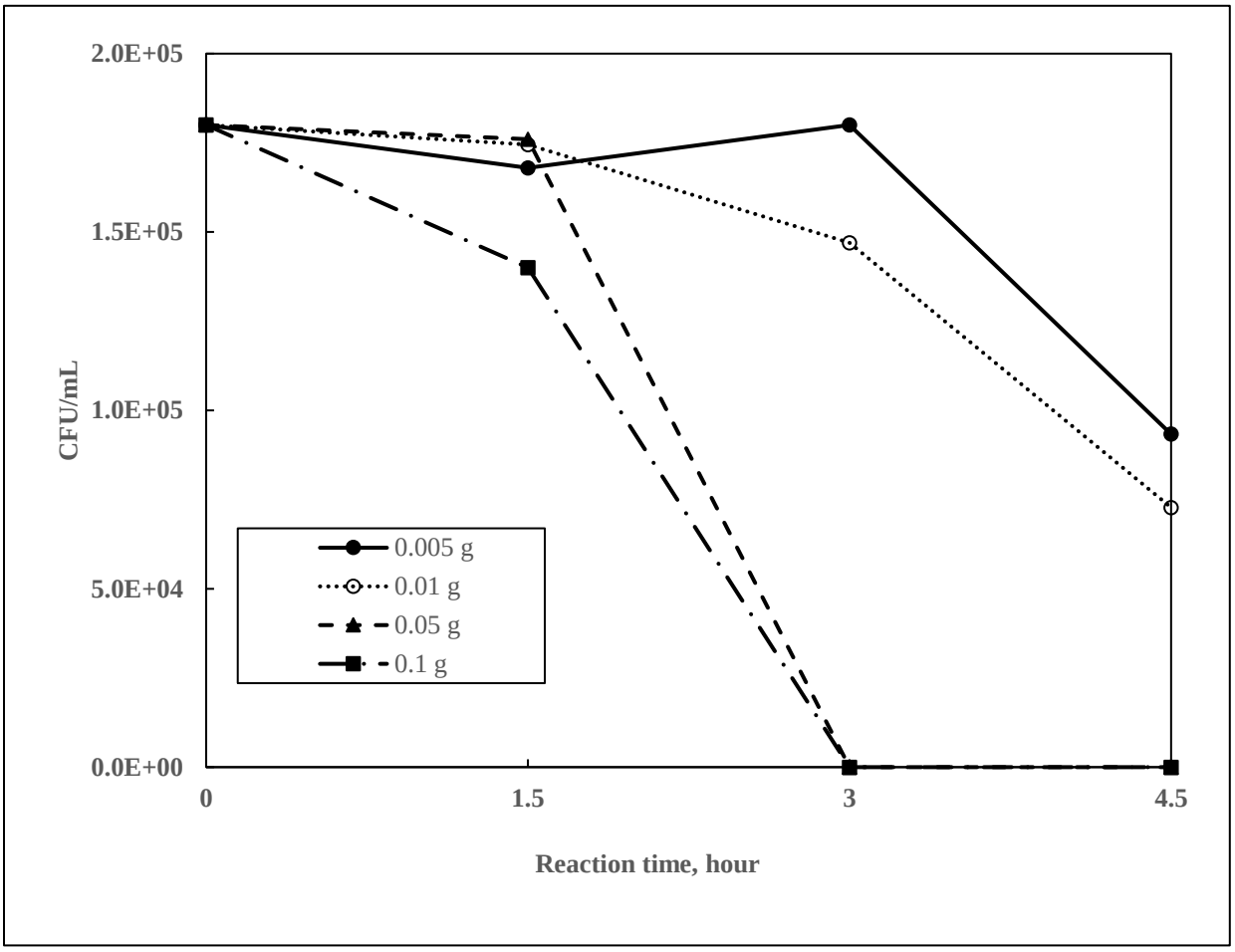


Figure 21. Effect of the amount of AIM against *E. coli* removal.

This study focused on *E. coli* inactivation mechanism of AIM, which has been investigated in our research group with interesting findings. The main advantage of making ZVI into AIM is controlling ZVI surface passivation by changing iron oxides and coating magnetite on the surface of ZVI. The AIM technology has been commercialized in wastewater treatment mainly focusing on heavy metal removal. In the previous study, we demonstrated the effect of

AIM on bacterial removal and confirmed that there were both of bacterial adsorption and inactivation. Although the exact chemical mechanism of singlet oxygen generation remains uncertain, the most remarkable finding of this study is that singlet oxygen can be generated by AIM without external photo energy, and the singlet state oxygen is the main reason for bactericidal effect of AIM system.

III.6 Conclusion

This study focused on the mechanism of bacterial reduction caused by AIM oxic reaction in water system. The following conclusions can be drawn:

- AIM could significantly reduce *E. coli* in water system.
- Singlet oxygen scavenger inhibited the bacterial removal effect of AIM, but other scavenger showed negligible effect.
- Singlet oxygen scavenger, SA, did not affect absorption onto a surface of the media.
- According to the 2) and 3), it can be inferred that singlet oxygen is the main reason for bacterial inactivation in AIM system.
- Magnetite coating on the surface could keep a reactivity of core ZVI, and this could be a key factor for exciting singlet state by chemical energy.

III.7 Summary

Active Iron Media (AIM) or mixed ZVI/Fe_3O_4 media system is a result of ZVI preconditioning which is made by additional $Fe^{2+}$and NO_3^- in anoxic condition. Previous study demonstrated that AIM media effectively removed bacteria in the aqueous system. This study is to discover bacterial inactivation mechanism in terms of reactive oxygen species (ROS) generation during oxic reaction of the AIM media. ROS scavenger assay was performed in order to elucidate the effect of ROS generation on bacterial inactivation. As a result, singlet oxygen scavenger, sodium azide, significantly inhibited the bactericidal effect of AIM in both bulk and solid phase. Singlet oxygen was measured by quantifying a furfuryl alcohol (FFA) concentration and the concentration of singlet oxygen increased with a reaction time of AIM and the concentration of additional Fe^{2+}. However, pure ZVI (without preconditioning) did not produce a singlet oxygen even in the presence of the highest concentration of Fe^{2+}. The Fe^{2+}/Fe^T ratio in the aqueous phase with AIM at the end of the reaction was significantly lower than that of normal ZVI reaction. The results of this study suggest that the bactericidal effects of AIM is caused by singlet oxygen generation in a consequence of redox of AIM in oxic condition, and magnetite (Fe_3O_4) which is coated on the surface of ZVI has a crucial role in singlet oxygen generation.

CHAPTER IV

IMPACT OF DAIRY MANURE PROCESSING USING POLYACRYLAMIDE ON ANTIBIOTIC RESISTANT BACTERIAL LEVEL*

IV.1 Introduction

In rural areas near dairy or other animal farming facilities, groundwater is the most common source of drinking water (Kenny et al. 2009; Morris et al. 2003). Therefore, maintaining sustainable agricultural practices and developing science-based strategies for using antibiotics in dairy operations are of critical importance for protecting drinking water resources as well as food and worker's safety. However, such efforts are often hampered by a gap of understanding of bacteria occurrence and antibiotic resistance in dairy operations.

Applying antibiotics as a growth promoter at sub-therapeutic doses to cattle, swine, poultry, and even fish (Angenent et al. 2008; Kemper et al. 2008) is an essential part of the farm animal and fish production. Antibiotic consumption in modern livestock industry has increased significantly with one study reporting that the quantity of antibiotics used in 2004 was 108 times higher compared to that used in 1950 (Massé et al. 2014). About 91% of livestock facilities in the USA use over-the-counter antibiotics as growth promoters annually, amounting to a staggering 11.2 million kg antibiotics used annually (Sarmah et al. 2006; Shea. 2003; Arikan et al. 2009; National Research Council, 1999). A variety of antibiotic classes are used in the

*Reprinted with permission from "Impact of Dairy Manure Processing Using Polyacrylamide on Antibiotic-Resistant Bacterial Level" by "Sunghwa Han, Sharon C. Long, Troy Runge, Cuihua Dong, and Zong Liu, 2019. *Water, Air and Soil Pollution*, 230, 58, Copyright [2019] by Springer Nature

livestock industry, with the more common classes including β-lactams, Macrolides, and Sulphonamides (Mellon et al. 2001). The fate of these applied antibiotics ultimately becomes an animal waste issue, as the animals excrete a significant proportion of those antibiotics, 17-90% for livestock (Bound and Voulvoulis 2004; Kumar et al. 2005; Pathak 2010), directly through their urine and feces, unchanged or as active metabolites such as epimers or isomers of the parent compound (Mackie et al. 2006). Antibiotic use, including treating sick cows on dairy farms, has contributed to the spread of antibiotic resistance as a consequence of the release of antibiotics, antibiotic resistant bacteria, and antibiotic resistance genes into the environment (Martinez 2009), which could cause antibiotic-resistant bacteria presence in dairy manure (Esiobu et al. 2002). Dairy manure is an excellent soil conditioner and fertilizer because of its organic carbon content originating from undigested lignocellulosic fiber, nitrogen content from urea and degraded proteins, in addition to the essential nutrients for plants growth, such as phosphorus (Liu et al. 2016). However, larger farms are increasingly processing manure before land application in order to meet lower hauling costs, decrease environmental concerns around nutrient losses to water, and satisfy federal and state regulations. Therefore, a comprehensive manure handling and treatment strategy is crucial on a large dairy farm. A typical manure management strategy is liquid/solid separations through a variety of operations such as screens, presses, or centrifuges to produce a nutrient-rich in solids and a low-nutrient/solids in liquid stream. The properly separated liquid can be used for irrigation or recycled to use as process water to flush the barns. Closing the waste cycle on dairy farms worldwide can increase the sustainability of such operations; however, research into factors that could lead to “unforeseen” consequences from changes in management practices is essential to ensure the efficacy of such changes.

On large farms, physical separation methods such as centrifugation are often enhanced by chemical addition (Vanotti et al. 2002; Amuda and Alade 2006; Liu et al. 2017) because physical separation alone is not suitable to remove fine suspended particles, which typically contain the majority of the nutrients, from recycled liquid streams (Liu et al. 2016). These chemicals bind and separate the smaller particles for efficient concentration of solids and nutrients (Zhang and Westerman 1997). For example, the use of polyacrylamide (PAM) polymers, their homo-polymers, and their acrylamide/acrylic acid co-polymers, alone or in combination with various inorganic salts, have proven to be effective in enhancing concentration of solids and nutrients in the separation process (Vanotti et al. 2002). Despite the efforts of recent studies examining manure separation, there is still a lack of studies relating polymer effect on pathogen indicator reduction, as well as dairy manure characteristics such as the level of total solids, chemical oxygen demand (COD), and liquid-solid separation efficiency of raw manure. Furthermore, there is little information of the effects of polymers on bacterial concentration and especially on antibiotic resistant bacteria in a raw manure and in the liquid stream of polymer treated manure. The objectives of this study were to evaluate the occurrence of antibiotic resistant bacteria in a raw dairy manure and determine the effect of commercial polymer treatment on the removal of those resistant bacteria in a raw manure for potential liquid reuse or recycling. In this paper, we evaluated the effect of manure treatment with polymer coagulation/flocculation on liquid phase pathogen indicator reduction. In addition, the occurrence of antibiotic-resistant bacteria among total live bacteria against four different types of representative antibiotic typically used in dairy facilities (tetracycline, penicillin, florfenicol, and cephalosporin) was evaluated by an antibiotic scoring method which was developed from this study.

IV.2. Materials and Methods

IV.2.1 Sample Collection and Reagents

Raw manure samples used in this study were collected from a dairy farm in eastern Wisconsin. Sample collection was conducted at several sampling points using 250 mL freshly sterilized polypropylene containers and then promptly transferred to a sealed cooler with ice. The inside temperature of the cooler was kept at 4℃ before analysis. A bacterial analysis was performed within 12 hours after we collected the raw manure samples. Remaining samples after bacterial analysis were kept frozen in -20℃ freezer until used for other experiments. To conduct chemical experiments, frozen samples were thawed at room temperature.

All reagents used in this study were reagent grade unless otherwise specified and stored through manufacturer's instructions. The polymer reagents (cationic polyacrylamide) were obtained from Soil Net LLC, Belleville, WI. A total of 8 polymer samples were evaluated in this study for manure separation, coagulation, and flocculation. Polymer stock solutions were prepared freshly for every experiment. Each polymer sample was diluted to 1% (v/v) with deionized (DI) water with mixing. The mixed emulsion was added to manure at a ratio of 3:100 (v/v).

IV.2.2 Manure Treatment and Separation

For the solid settling time/velocity test, jar tests were performed in order to evaluate the raw manure clarification efficiency of each polymer. The jar test was divided into a control group (raw manure) and an experimental group (centrifuge with/without polymer addition) and performed by the following procedure. Identical 400 mL low form Griffin beakers were used for the jar test. Six mL of diluted polymer emulsion of each polymer was applied to each of 200 mL manure sample. The manure samples were mixed using a magnetic stir bar on stir plates and the

settling time was measured. Measurement began after the mixing was stopped. The settling distance was measured at desired time intervals using a calibrated graduated scale (minimum detection limit of 0.5 mm) marked on each beaker. The clarification efficiency was calculated using the volumetric ratio of clear liquid volume (after polymer treatment) to the initial volume. The solids content of manure samples was measured gravimetrically in accordance with American Public Health Association (APHA) Standard Methods 2540 (2005).

IV.2.3 Bacterial Indicator Analysis

Total coliforms and *Escherichia coli* (*E. coli,* a subset of total coliforms that are closely associated with mammalian fecal matter), are frequently used as indicators of the potential for pathogen presence in animal manure since they are typically present in higher densities than any single pathogen (Garzio-Hadzick et al. 2010). In this study, the Colilert™ method (IDEXX, Westbrook, ME) was used for simultaneous detection of total coliforms and *E. coli*. A non-nutritive but biologically gentle matrix (Dilu-Lok phosphate buffer with magnesium chloride, Hardy Diagnostics, Santa Maria, Cal.) was used for dilution. To enumerate the cells, each sample was serially diluted by applying 1 mL to 99 mL of buffer, making a 10^{-2} dilution of the original sample. Up to 10^{-8} of subsequent serial dilutions were applied as needed in order to estimate a detectable concentration of the bacteria. A packet of Colilert™ reagent was added to the diluted samples and well mixed. The entire content of each sample was poured into a Quanti-Tray/2000 (IDEXX Laboratories, Westbrook, ME), sealed, and was then ready for incubation. The trays were incubated for 24 to 28 h at 35°C ± 0.5°C. After incubation, the results were examined by observing and counting the wells; yellow color for total coliforms; and fluorescence emission under ultraviolet light for *E. coli*. The most probable number (MPN) of total coliforms and *E.*

coli was determined according to the manufacturer's instructions and was based on the statistical Poisson distribution of positive and negative wells.

IV.2.4 Antibiotic Resistance Test

Four representative antibiotic classes (Tetracycline, β-lactams (Penicillin), amphenicols (Florfenicol), and Cephalosporin) were chosen for the antibiotic resistance evaluation since those four classes are the most commonly used in a dairy farm management. Lower and higher dose of each antibiotic was applied based on their known minimum inhibitory concentration (MIC) values in order to estimate the percentage of antibacterial resistant bacteria in a raw manure and separated manure samples. The concentration information of each antibiotic reagent is summarized in Table 2. For estimating a ratio of antibiotic resistant bacteria, 1 mL of raw manure and a liquid stream of centrifuge/centrifuge with polymer separated samples were inoculated into a normal tryptic soy agar (TSA) plate and TSA plates containing lower and higher concentration of each antibiotic and incubated in 35℃ for 24 hours. After incubation, colonies of the antibiotic containing plates were counted and compared to those of the control TSA plates.

The resistance levels of the tested bacteria were indicated using a scoring method. Every single colony on the antibiotic containing plate was transferred to 8 individual media (a lower and a higher concentration of each of the 4 different antibiotics). Each of the eight media were poured into a 100 x 15 mm and 6 x 6 square grid TSA plate, each single colony was replica transferred and all plates were incubated in 35℃ for 24 hours. For example, a single colony from a 12.5 ppm TSA plate was taken and transferred to each plate containing the four different antibiotics of lower and higher concentrations. After incubation, the colonies on each space were counted. The result was indicated by a score of 0 or 1 for absent or presence of growth,

respectively. The score range of each colony (bacterial strain) would be between 0 to 4 since we did not double count if a colony was formed in both of lower and higher concentration plates of the same antibiotic.

IV.3 Results and Discussion

IV.3.1 Separation efficiency of PAM

Dairy manure with high solids (higher than 4%) content can take several weeks to months to separate into high-solid and low-solid phases in settling tanks without any flocculent/coagulant addition or other treatment such as without accelerated separation such as centrifugation. In the previous study (Liu et al. 2017), screening tests were conducted with a variety of PAMs in order to select a suitable polymer for optimum solid-liquid separation of raw manure. The efficiency was indicated as a clarify efficiency (%) of the liquid stream after each desired settling time. The polymer screening results suggest that several PAM polymers had a significant effect on improving the manure separation and settling characteristics. PAM 3 was chosen as for the remainder of experiments since PAM 3 showed the best separation efficiency among 8 PAM samples used in this study as shown in Fig. 22. Results of control group represented that no separation was observed within 1 h settling without any polymer additive. As high as 28% of separation efficiencies were achieved within 1 h when polymers were added.

IV.3.2 Effects of polymer treatment on solids content and bacterial reduction

Bench-scale centrifugation study was conducted for a better understanding of the effects of a centrifuge and chemical additives on manure solids separation and bacteria reduction. Because of the low solid-separation and bacterial indicator level reduction efficiencies of large scale centrifuge, higher speed centrifugation and longer retention time were applied in the lab scale experiments. In the previous study, the effect of the centrifuge on bacterial reduction was investigated (Liu et al. 2017), and we concluded that the centrifugal speed of 2,000 g does not affect bacterial concentration significantly. Therefore, we selected 1 minute centrifuge time with

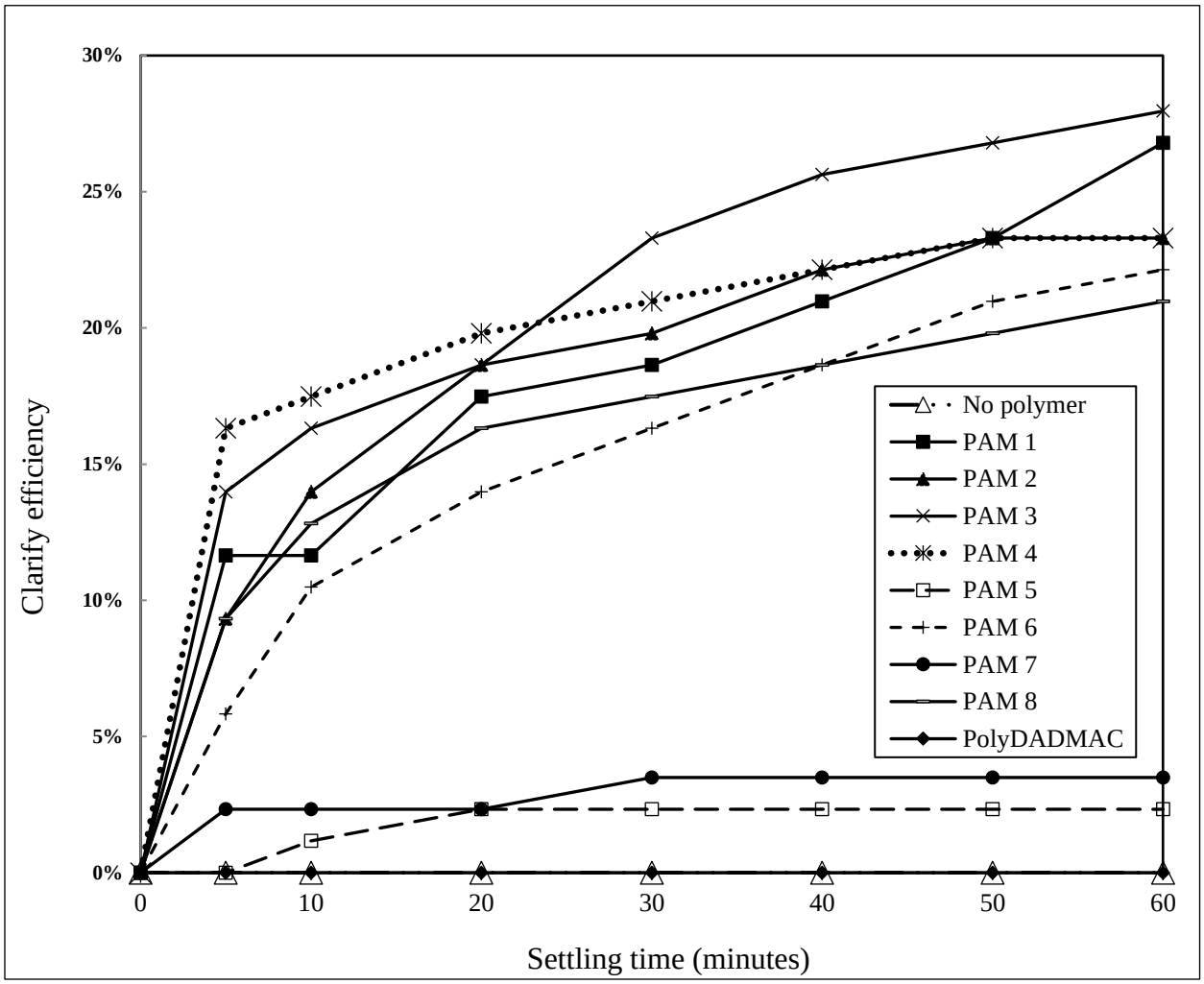


Figure 22. Liquid-solid separation efficiency by different polymers.

a speed of 2,000 g to evaluate the sole effect of PAM, excluding effects of the centrifuging, on solids content and bacterial reduction.

Results presented in Table 1 indicated that PAM 3 has an effect on reducing solids content and bacterial concentration in the liquid stream of raw manure. The result showed that the PAM 3 treatment significantly reduced solids content in the resultant liquid, which means

that the PAM 3 has a crucial impact on liquid-solid separation of raw manure. The PAM 3 used in this study was determined to possess a low charge density (0.93 meq/g) high molecular weight (6000 kDa) cationic polymer with 88.8% solids content. Since most of natural organic matter or bacteria are known to have a negative charged at neutral pH, the positively charged polymer could effectively coagulate the suspended solids in the liquid stream (Liu et al. 2016; Liu et al. 2017).

Treatment	**Solids Content (%)**	**Bacterial Indicator (MPN/mL)**	
		Fecal coliform	*E. coli*
Raw Manure	3.11 ± 0.036	48,800	35,000
Centrifuge w/o PAM 3	3.01 ± 0.008	49,500	38,400
Centrifuge w/ PAM 3	2.17 ± 0.137	30,100	22,600

Table 1. Effects of PAM treatment of raw manure on solids content and bacterial indicator level.

In addition, the high molecular weight of PAM may be operating through a bridging flocculation mechanism in addition to charge neutralization. Flocculated materials which are formed via bridging flocculation stay apart when broken up since polymer tails and loops

bridging across two or more particles are physically separated apart by the shearing forces (Wong et al. 2006).

The effect of PAM 3 on bacterial reduction is summarized in Table 1. The treatment of PAM 3 removed 61.7% and 64.5% of fecal coliforms and *E. coli*, respectively, from the liquid stream. In a previous study, we confirmed that there was no significant effect of PAMs on changes in the number of *E. coli* cells in the nutrient rich condition, even at the highest concentration of the polymer dosage (Liu et al. 2016). Therefore, this result suggests that PAM 3 could reduce bacteria in the liquid stream of raw manure by coagulation and flocculation process without affecting the viability of the bacteria. The centrifuge without PAM treatment under our experimental conditions did not decrease bacterial indicator levels (Table 1), which confirms that PAM 3 has a major role in bacterial removal.

IV.3.3 Analysis of antibiotic resistant bacteria

The information and results of antibiotics, concentration, and the effect of PAM treatment on antibiotic resistant bacterial concentration is summarized in Table 2. The antibiotic resistant bacterial percentage was represented as the ratio of the number of colonies on the antibiotic containing TSA plate against number of colonies on the normal TSA plate. The result showed that raw manure has 37%, 17%, 20%, and 33% of antibiotic resistance bacteria against tetracycline, penicillin, florfenicol, and cephalosporin, respectively. The results were only taking account for the aerobic bacteria which can be grown on tryptic soy medium.

As shown in Table 1 and 2, PAM treatment decreased the total number of bacteria in raw manure, however, the percentage of antibiotic resistant bacteria increased for all types of antibiotics. The increase of antibiotic resistant bacteria could be caused by the attenuation of

antibiotic levels by an effect of polymer coagulation. Choi et. al, reported that the effect of coagulation on the antibiotics in water (Choi et al. 2008). For example, functional groups of

Antibiotics	Concentration	Antibiotic resistant bacteria (%)		
		Raw	PAM	w/o PAM
Tetracycline	12.5 μg/mL	37	46	34
	25 μg/mL	40	23	31
Penicillin	100 U/mL	17	35	17
	200 U/mL	23	19	7
Florfenicol	8 μg/mL	20	31	14
	64 μg/mL	3	0	0
Cephalosporin	2 μg/mL	33	54	59
	10 μg/mL	30	42	28

Table 2. Antibiotic resistant bacteria percentage in raw manure.

tetracycline are known as tricarbonyl, dimethylamine, and β-diketone. Tetracycline is negatively charged above pH 3 as a result of the dissociation constants of those functional groups (Qiang

and Adams 2004). Therefore, tetracycline in the raw manure should be present with a negative charge since the pH of raw manure is near neutral to weakly alkaline. Therefore, positively charged polymer can easily attract the tetracycline, results in the bacteria actually being exposed to a reduced amount of antibiotics.

Based on the results of this study, florfenicol showed the least number of antibiotic resistant bacteria in raw manure used in this study. Florfenicol is bacteriostatic, and its mechanism of action is similar to that of chloramphenicol (Keyes et al. 2000). The mechanism of resistance to florfenicol is unknown but is associated with the *flo* determinant, a highly conserved gene sequence detected in *Salmonella enterica serovar Typhimurium* DT104 (Bolton et al. 1999) and in the fish pathogen *Pasteurella piscicida* (*Photobacterium damselae*) (Kim and Aoki 1996). The *flo* gene confers resistance to both chloramphenicol and florfenicol (Keyes et al. 2000).

IV.3.4 Antibiotic resistant level scoring

Resistant bacteria (including multi-resistant bacteria), such as *E. coli*, *Pseudomonas aeruginosa*, *Acinetobacter* spp. and *Enterobacteriaceae* are present in many municipal wastewater plants (Kümmerer 2009). However, reliable data on production and consumption of the antibiotics are challenging to be estimated, as it varies with time and country (Bouki et al. 2013; Diaz-Cruz et al. 2003). It is agreed upon that major sources of antibiotic exposure to environment are from human excretion, farm animals, and direct disposal of medical and industrial wastes. Although some antibiotics are removed by natural degradation or sorption, not all antibiotics cannot be completely removed or degraded naturally (Batt et al. 2006; Giger et al. 2003). The antibiotic residue in the environment can be major route for the development of resistance in bacterial pathogens (Salters et al. 2004; Chee-Sanford et al. 2009). Therefore, there

are higher possibilities of presence of antibiotic resistant gene and bacteria in solid wastes and wastewater from the industries where are using plenty of antibiotics, such as animal farms. In a raw manure, it has been reported that half-lives of various antibiotics are shorter than the anticipated storage period of the manure, which can result in significant degradation of antibiotic molecules before land application (Boxall et al. 2004). However, the amount of antibiotic resistant bacteria and genes do not correspond to the concentrations of antibiotic compounds in the environment (Bouki et al. 2013). For example, β-lactams have been found in the environment at very low concentrations and they are easily hydrolyzed (Helland et al. 2010; Längin et al. 2009), whereas resistant bacteria and genes encoding resistance against certain β-lactams have been detected in municipal wastewater treatment plants (Kümmerer 2009). The presence of vancomycin resistant bacteria was reported in waters in Europe, even though only small quantities of vancomycin are used in the region (Kümmerer 2009). Therefore, measuring only residual antibiotics without an analysis of actual resistant bacteria will not be enough for understanding a profile of actual bacterial resistance levels. Land application of animal manure, with its high concentration of microbes, can be directly related to an introduction of new bacteria into the environment, including potential pathogens and some harmful viruses. The persistence and transport of these organisms in the environment continues to be a concern for environmental safety, food safety, as well as human and animal health. The longer an antibiotic persists in the soil in an active form, the greater the potential for native soil bacterial populations to be affected (Gavalchin and Katz 1994). For these reasons, it is essential to understand and estimate a contamination of antibiotics as well as antibiotic resistant bacteria.

In order to more deeply understand the antibiotic resistant bacterial levels, quantify the number of resistant bacteria in raw manure, and investigate a portion of multi-resistant bacteria,

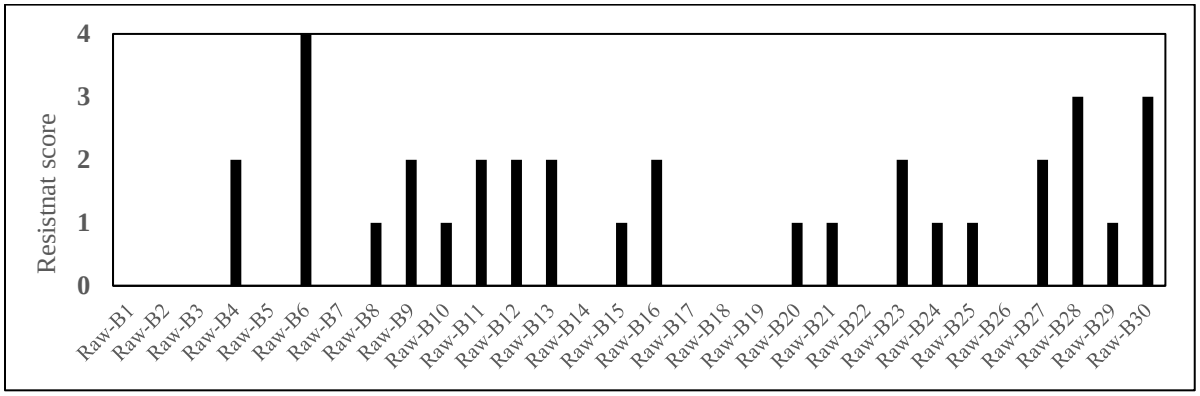
the scoring method was performed in this study. The number of total colonies on the TSA plates after incubation were 30, 26, and 29 colonies, for raw manure, PAM treated, and centrifuged sample, respectively. Each colony on the plates was transferred to 8 different 36 grid plates as described in the material and methods section, and the number of colonies after incubation were counted. For the scoring algorithm, we assumed each colony represents a different organism. That is, there are 30, 26, and 29 different aerobic bacterial strains in each group. It is recognized that in nutrient rich habitats, such as that of manure, the material may be colonized by clonal communities. However, this work is the first step in understanding the prevalence of antibiotic resistant bacteria in manure and the effects of polymer separation in controlling those populations. This simplifying assumption does not negatively affect our findings.

Fig. 23 summarizes the results of the antibiotic resistance scoring test. The average resistance score was 1.13, 1.65, and 1.28, for raw manure, PAM treated, and centrifuged group, respectively. The score of 2 or more represents that the bacterial isolates possess multidrug resistance. The result indicated that the number of antibacterial resistant bacterial isolates were 19 out of 30 in raw manure (66.33%), 17 out of 26 in PAM treated manure (65.38%), and 20 out of 29 in centrifuged manure (68.97%) against the four antibiotics used in this study. Among those resistant bacterial isolates, 11 (39.29%), 13 (54.17%), and 12 (44.44%) isolates demonstrated multidrug resistant, in the raw, PAM treated, and centrifuged manure, respectively. Overall, the liquid stream from PAM treated manure contained less bacteria in terms of the number of total bacteria, however, the remaining bacteria possessed a higher portion of antibiotic resistance. The reason for the higher percentage of antibiotic resistant bacteria and higher multidrug resistance in PAM treated manure may be explained by removal of the activity of PAM against antibiotics, as described previously.

IV.4 Implications

Use of antibiotics in humans and animals carries an inherent risk of selecting for antibiotic resistance genes. These genes are often found in bacteria with other genes promoting resistance to other potentially harmful chemicals (Alekshun and Levy 1999). The transmission of antibiotic resistant bacteria and genes from animals to humans has been demonstrated in the literature (Khanna et al. 2008; Smith et al. 2013). On-farm transmission of antibiotic resistance has been characterized in the literature for a wide range of animals. A recent review of the academic literature that addresses the issue of antibiotic use in agriculture suggests that only 7 studies (5%) argued that there was no link between antibiotic consumption in animals and resistance in humans, while 100 studies (72%) found evidence of a link (Singer et al. 2016). The other 32 studies (23%) presented the fact that the authors recognized the concerns of using antibiotics, but there was an uncertainty between usage of antibiotics and presence of antimicrobial resistance. Therefore, the degree to which the transmission from animals to humans, as well as the enumeration of bacteria in the real manure sample is of great interest and has significant implications for public and animal health.

(a)



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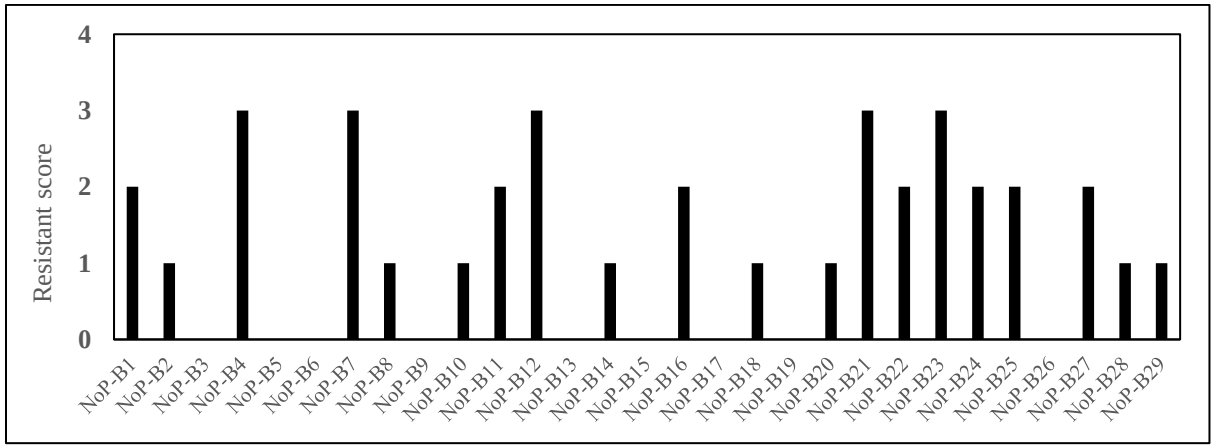

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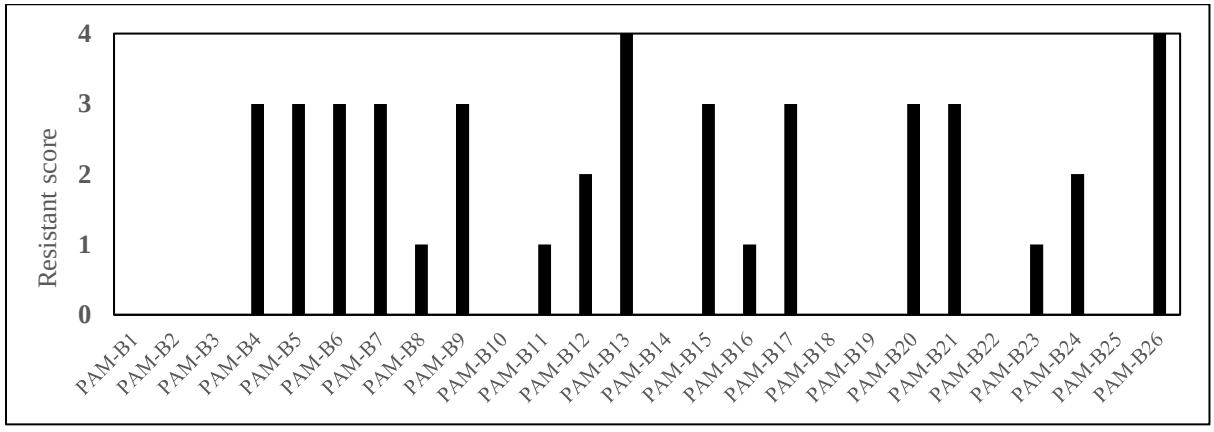


Figure 23. Antibiotic scoring of (a) non-treated raw manure; (b) centrifuged without polymer; (c) centrifuged with polymer.

If use of antibiotics in farm management is to continue at current or increased levels, appropriate treatment of the farm waste should be required to prevent discharge of the antibiotics, resistant bacteria, pathogens, and resistant pathogens. Application of PAM along with centrifugation could be one method to treat the animal waste since it showed an effect on bacterial populations in raw manure. In particular, the resistant level scoring test used in this study could be one factor that represents an antibiotic resistant level and will help to understand the presence of antibiotic resistant bacteria in a farm water system. Although, further studies should be conducted such as identification of resistant bacteria or genetic studies for resistant bacteria, this study can give a direction for an appropriate treatment of raw manure.

IV.5 Conclusion

Effects of PAM addition with centrifuge were investigated in this study using a systematic factorial experimental design. The impacts of both centrifugation and polymer addition on lowering the bacterial indicator levels in the liquid stream of the manure were measured. PAM 3 significantly increased manure liquid/solid clarification fraction but had negligible effects on solids reduction and indicator bacteria reduction when centrifuged was applied without polymer addition.

A percentage of antibiotic resistant bacteria in manure sample was investigated by comparing the number of colonies on agar media containing with/without 4 different types of antibiotics that commonly used in a real farm. 65.38% of aerobic bacterial isolates in the raw manure showed antibiotic resistance against antibiotics used in this study, and 40% of total bacterial isolates showed multidrug resistance. The results from this study suggested cationic polymer treatment could be considered during manure solid/liquid separation if improving pathogen reduction is a concern on the farm. Further investigation using genetic approaches in conjunction with polymers known to have biocidal activity or effect of polymer on the reduction of antibiotics could lead to improved and more effective manure processing and recycling approaches.

IV.6 Summary

This study investigates levels bacteria through population indicators as well as the levels of antibiotic-resistance bacteria in dairy manure. Although overall bacteria levels may be reduced during manure processing, it is of interest whether changes in management practices could lead to increased levels of antibiotic-resistance bacteria, which are becoming more prevalent in agricultural soils, groundwater, and surface water. Appropriate manure treatments are needed not only to reduce the potential risk of exporting antibiotic resistant bacteria to an environment, but also reduce antibiotic resistant bacteria exposure to animals if processed water is recycled. Results from this research revealed manure separation under relatively low speed centrifuge with 100 ppm polyacrylamide (PAM) emulsion addition reduced bacteria indicators population such as total coliforms and *Escherichia coli* (*E. coli*) significantly in the liquid stream compared to no PAM added. However, the percentages of antibiotic resistant isolates in liquid stream after centrifuge with PAM were higher compared to raw manure and no PAM added. Antibiotic resistance (cephalosporin, florfenicol, penicillin, or tetracycline) was observed or 65.38% of bacterial isolates in manure from a large dairy farm in Wisconsin and 39.29% of isolates demonstrated multidrug resistance. The results from this study strongly suggest that appropriate manure treatment is essential in order to help minimize the abundance of antibiotic resistance in our water environment.

CHAPTER V

SYNERGISTIC EFFECTS OF HIGH POSITIVE CHARGED POLYMER AND HYDROGEL ON BACTERIA INDICATOR REDUCTION

V.1 Introduction

Infections by pathogenic microorganisms are of great concern in many fields. These infectious diseases could kill worldwide more people than any other single cause (Muñoz-Bonilla and Fernández-García. 2012). In particular, in agricultural field, pathogenic contamination could be worse because manure has been used often as a fertilizer. Treating and utilizing manure can be a challenging mission for modern animal feeding operations specialized in intensive production. These systems produce a considerable excess of manure, which has a high risk of becoming a source of air, water, and soil pollution, especially it could be a major source of bacterial contamination of our environment (Liu and Wang, 2020).

Solid/liquid separation of the manure have become a common process in manure treatment since this process produces a nutrient-rich solid, a low-nutrient, and low solids liquid stream, which is a desirable condition in manure management (Vanotti et al. 2002). Mechanical separation alone is not suitable to remove fine suspended particles since raw manure typically contains a majority of the nutrients; thus a fair amount of nutrients remains in the liquid stream unless additives are used to enhance their removal (Liu et al. 2016). Chemicals to flocculate the smaller particles are used to effectively concentrate manure solids and nutrients during separation (Szögi et al. 2006). Charged polymers are often used as a coagulant/flocculant in the agricultural wastewater treatment.

Typically, the polymer addition has some advantages such as lower dosage requirement and less environmental impact compared to conventional chemical coagulants (i.e. $Fe_2(SO_4)_3$ and $Al_2(SO_4)_3$) (Liu et al. 2016). There has been a trend in using polyacrylamide (PAM), its homopolymers and its acrylamide/acrylic acid copolymers to effectively separate solids from wastewater (Garcia et al. 2007). It is well-known that most bacterial cell walls are negatively charged in standard condition (Han et al. 2019) because it contains phosphatidylethanolamine as the major component; therefore, the polymers that have an antibacterial effect are mostly cationic (Muñoz-Bonilla and Fernández-García. 2012). In this reason, polymers have a quaternary ammonium functional groups are well studied in terms of its biocidal characteristics (Kenawy et al. 2002).

It is generally accepted that the mechanism of the bactericidal action of the polycationic biocides involves destructive interaction with the cell wall and/or cytoplasmic membranes (Liu et al. 2016; Kenawy et al. 2002). Agar is a heterogeneous mixture of two polysaccharide components: agaropectin and agarose, which share the same galactose-based backbone (Williams and Phillips. 2000). Agaropectin is modified with acidic side groups, such as sulphate and pyruvate, while agarose has neutral charge and possesses longer chains (Freile-Pelegrin and Murano. 2005; Blanco-Fernandez et al. 2011).

Agar has been being used not only for experiment but also for food, pill coating, cosmetics, and so on. However, the use of agar is barely used for water treatment, and there have been no study of the synergistic effect of agar combined with polymers. The purpose of this study was originally to investigate the water treatment efficacy and bacterial removal efficiency of polymer when it applied with hydrogel in a gel form. However, during the course of the research, we accidently discovered a synergistic effect of polymer on bacterial removal and/or

inactivation, when it is applied with hydrogels such as agar and/or agarose. Therefore, the purpose of this study is to study 1) the antibacterial performance of polymer, 2) the study of water-treatment of polymergel, and 3) the synergistic effect of hydrogel and its components with polymer on bacterial removal.

V.2 Materials and Methods

V.2.1 Reagents

All reagents used in this study prepared or diluted with deionized (DDI) water (E-pure D4641, USA) and stored by following manufacturer's instruction. Polymer samples used in this study were obtained from *SnF* Holdings Co., Inc. Normal saline solution was prepared with 0.85% of $NaCl$ (BDH, USA) in deionized (DI) water, followed by autoclave sterilization in 121°C for 15 minutes and stored in 4°C before use. All chemicals used in this study were analytical reagent grade.

V.2.2 Polymer and polymer gel sample preparation

Polymer samples used in this study were emersion type and some of them were not easily diluted with water. Thus, samples should be diluted first for conducting experiment using polymer. Polymer samples were diluted 1% (v/v) first by adding 1 mL of each polymer sample into 99 ml of autoclaved DI water with rapid mixing (greater than 600 rpm). At least 10 minutes of mixing time were given to each polymer samples for complete mixing. After polymers were diluted and mixed, each sample was further diluted with autoclaved DI water to desired concentration. Autoclaved 125 mL flasks were used as a reactor.

Agar and Agarose were used as gelling agents for making hydrogel in this study. Concentration of 0.5 - 3% (w /v) of agar or agarose were used to make hydrogel containing polymer (poly-gel). To make poly-gel, desired concentration of agar/agarose powder were added to DI water, followed by autoclaving the gel for dissolving and sterilization. The liquefied hydrogel was cooled down at room temperature until it reached 40 - 45°C. Desired amount of the prepared polymer samples (1%, v/v) were added to the liquefied gel to make target concentration of polymer. The gel – polymer mixture was then gently shaken by hand to make the polymer gel

homogeneous and to prevent foaming. Well mixed poly-gels were solidified at room temperature for at least 1 h. Final volume of the gel was 20 mL.

V.2.3 Bacterial experiment

V.2.3.1 *E. coli* isolation, identification, and preparation

E. coli was isolated from raw dairy manure collected from regional livestock research facility in central Texas. The isolation and identification of the bacteria used in this study was performed in the previous study and the detailed procedure is described in the Chapter III. The isolated bacteria was sub-cultured daily on the Trytic Soy Agar (TSA, Becton, Dickinson and Company, USA) media. Bacterial stock was prepared with 60% sterilized glycerol and stored at -20°C for future use. For activation and enumeration of isolated strain, single colony from the agar plate was collected and transferred to Tryptic Soy Broth (TSB) media and incubated at 37°C for at least 16 h before each set of experiments. Bacterial concentration was determined by measuring optical density with spectrophotometer at wavelength of 600 nm (1.0 at OD_{600} equals to 8×10^8 colony forming unit (CFU)/mL).

V.2.3.2 Effect of Polymer-gel on bacterial removal

To estimate the effect of poly-gel on bacterial removal, 50 mL of normal saline solution was added on the gel to make final volume of 70 mL (20 mL of gel and 50 mL of normal saline). Subsequently, the bacterial suspension was serially diluted with normal saline solution (0.85% NaCl) and inoculated into the water. Final concentration of *E. coli* in each reactor was 5~10 × 10^6 CFU/mL. Reactors were shaken at 200 revolutions per minute (rpm) for desired reaction time (~3 h) at room temperature. After desired reaction time, liquid phase of each reactor was collected. Collected samples were serially diluted with normal saline solution and the live cell

concentration was measured by the pour plate method. The bacteria reduction experiments were all performed in duplicate and repeated at least three times.

V.2.4 Synergistic effect of Polymer with hydrogel on bacterial removal

To evaluate a synergistic effect of polymer with a gel component, the bacterial reduction abilities were compared between following experimental group: blank (bacteria only); control (polymer addition only without gel); polymer-gel phase (hydrogel containing polymer); and polymer-gel-liquid phase (liquid phase after 24 hour of polymer-gel release).

To make polymer-gel-phase and polymer-gel-liquid phase, concentrations of 0.5 - 3% agar/agarose containing amount of 0.5 - 5 mL polymer were prepared followed by addition of 50 mL of normal saline solution. Each reactor was given by reaction time of 24 hr in room temperature for polymer release. After 24 hour of releasing time, the half of liquid phase (25 mL) from each reactor was collected (polymer-gel-liquid phase) and the remaining 25 mL liquid + polymer-gel was considered as polymer-gel phase. *E. coli* was inoculated to these two group at the final concentration of 2-5 x 10^5 CFU/mL.

In addition, to deeper understanding of the synergistic effect of the hydrogel on bacterial removal, a comparative experiment was also conducted on the agar-release control / agar-release-polymer-addition group. Agar (or agarose) gels with a concentration of 0.5-3% without addition of polymer were prepared first and then shaken for 24 hours to allow the components of agar (or agarose) to soak in water. After that, only the liquid phase was separated and collected. Polymer was added to this liquid phase to prepare the [gel-release-polymer addition group]. *E. coli* were inoculated into these two groups and bacterial removal efficiency was compared. Since the main component of agar / agarose used in hydrogel is polysaccharide, the synergistic effect

of sucrose (at the same concentration as gelling agent) and polymer was also compared in parallel with the above experiments.

V.2.5 Evaluation of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

In vitro susceptibility tests were performed in sterilized 15 mL centrifuge tube to determine MICs of polymer samples against *E. coli*. The MIC and MBC were evaluated using the modified method described in the guidelines of CLSI M7-A6. Two-fold dilution of polymer was performed to make a different concentration range of polymer (512 to 0.5 ppm (v/v)). Two control reactors: one contains only bacteria without polymer, and the other is blank (only bacteria) were compared to see bacterial contamination, and bacterial condition, respectively. Reactors were incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of sample that resulted in the complete inhibition of visible growth. Normal saline solution was used for bacterial dilution. MBCs were determined by removing the bacterial suspension from each of MIC reactor that showed no visible growth and sub culturing onto TSA plates. The plates were incubated at 37°C for 24 h until growth was seen in the growth control plates. MBC was interpreted as the lowest concentration of sample that showed no growth of bacteria.

V.2.6 Statistical analysis

Experiments were performed at least in three times throughout the study and all data are presented as the mean ± standard error of repeated values. Statistical significance was determined using Student's t-tests by evaluation of differences by comparing to control or blank groups and analysis of variance (ANOVA) test; $*p < 0.05$ or $**p < 0.01$ were considered statistically significant based on the tests. JMP pro 13 was used as a software tool for statistical analysis.

V.3 Results and Discussion

V.3.1 Effect of polymer gel on *E. coli* removal and selection of final candidate

Fig. 24 shows the results of removal efficiency of polymer-gel containing 39 different polymer samples against *E. coli*. As shown in Fig. 24, the bacterial removal performance of polymer gel varied from 0 to 6.85 log scale reduction when compared to control group. The 6.85 log reduction indicates complete removal at this experimental condition (as the final bacterial concentration of the control group after 24 hr reaction was 6.85 log). Among the three different polymer types (polyacrylamide, polyamines, and polyDadmac) used in this study, polyamine showed the best bacterial removal performance, followed by polyDadmac and polyacrylamide. The relative molecular weight of each polymer could be estimated by the number in each polymer name. For example, in the polyamine group, the FL 3250 has higher molecular weight than FL 2250. Although the relationship between polymer molecular weight and bacterial removal efficiency is not perfectly linear trend, but higher molecular weight polymers tend to exhibit higher bacterial removal performance. According to the screening result, the final candidate we chose in this study was FL3050 in the polyamine group. FL4620 and FL4520 from polyDadmac, and FL3249 and FL2650 from polyamine showed similarly high bacterial removal, but the FL3050 had following two major advantages; 1) FL3050 showed the largest removal efficiency against phosphorus (data not shown), which is the largest water quality issues in the agricultural field. 2) because of the lowest viscosity, it was the easiest to handle. These characteristics can be a great advantage not only in the experimental condition, but also in applications.

The alphanumeric code following the number of each polymer name indicates the branch type of the polymer molecular structure and the degree of different positive charge.

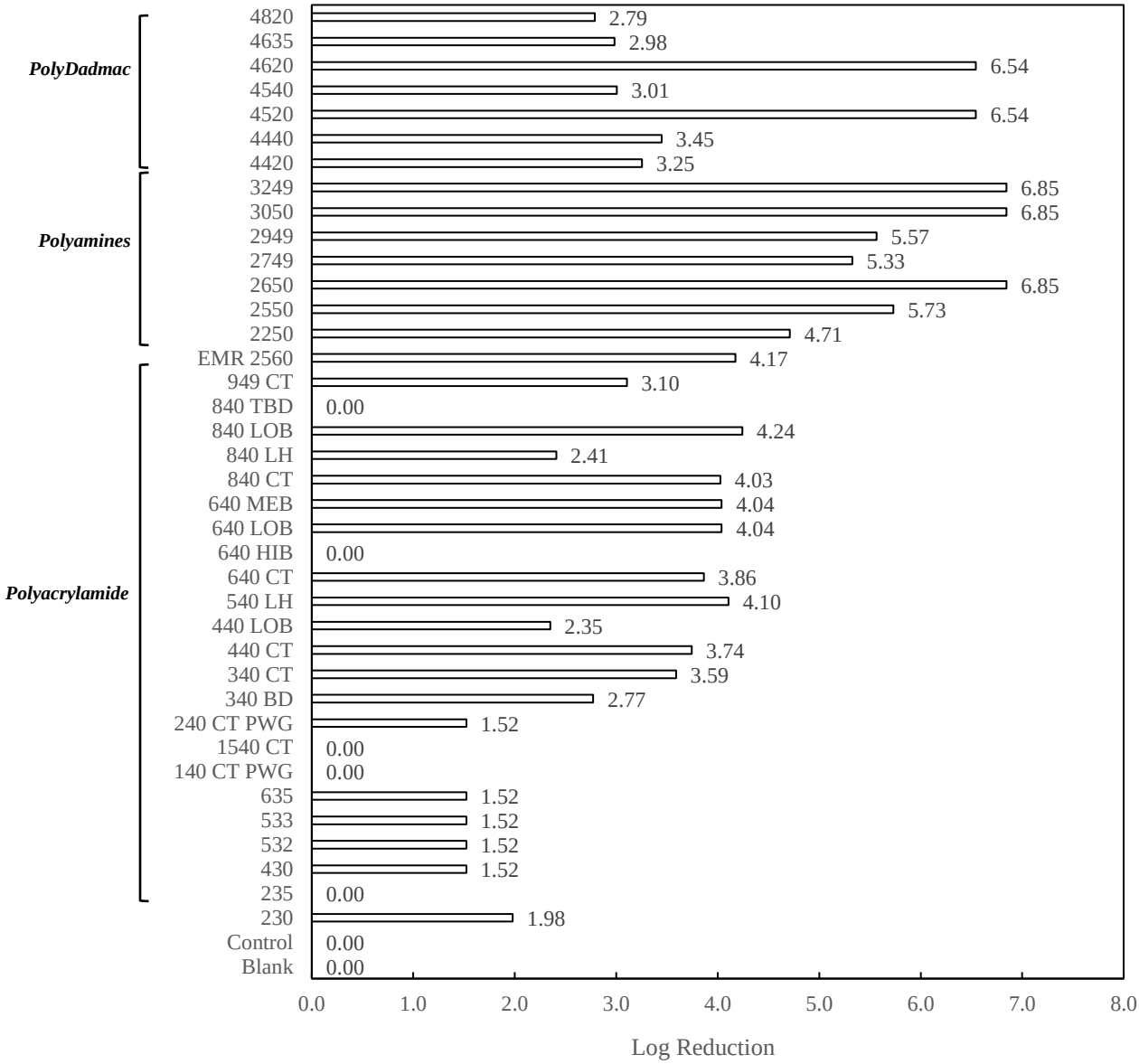


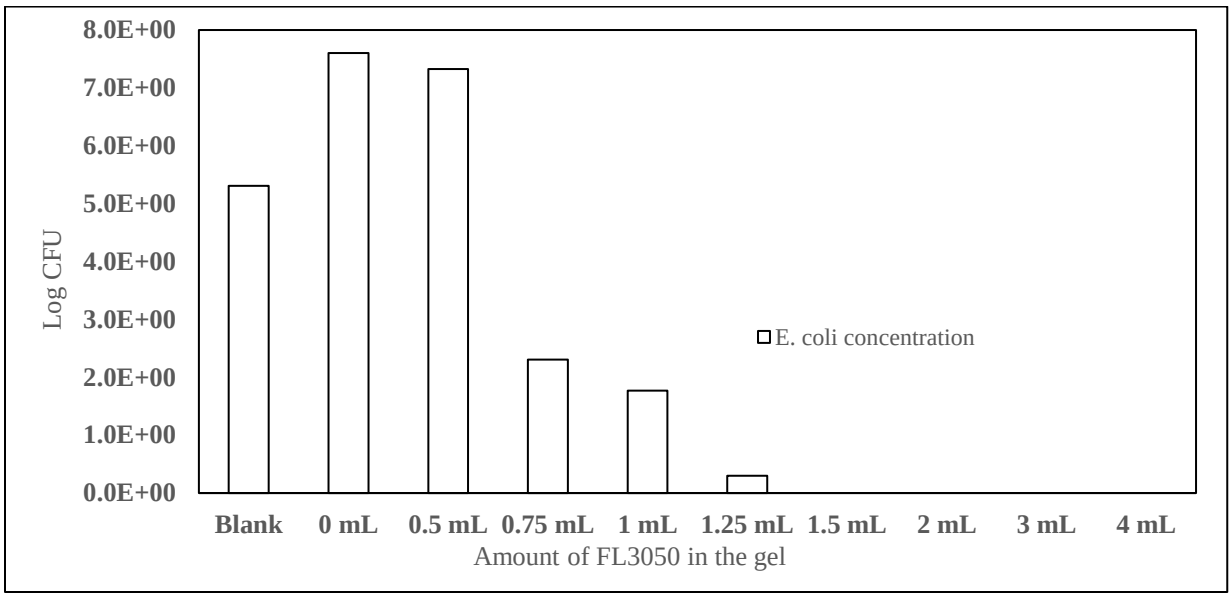
Figure 24. Effect of polymer-gel on *E. coli* removal. 39 different polymers of different types (PolyDadmac, Polyamines, and Polyacrylamide) were used to make polymer gel and their bacterial removal efficiency were evaluated.

The exact information about physical and chemical properties of the polymer cannot be provided in this article because it is confidential. However, it can be concluded that the branch of the polymer could have a huge impact on the bacterial removal activity. For example, for polyacrylamide 840, there are four samples which have different branch to the polymer (TBD, LOB, LH, and CT). LOB and LH showed considerably higher bacterial removal activity, but bacteria were not removed at all for TBD sample (Fig. 24). The effects of molecular characteristics on bacterial removal might be important data for the study of the bacterial removal mechanism of polymers, but it will not be consistent with the scope of this study. The bacterial removal in water system could be considered as physical action such as sedimentation by coagulation or bacterial inactivation caused by cytotoxicity of the agent used for water treatment. For the cationic polymer, previous studies have demonstrated that polymer itself has a bactericidal effect.[4]

V.3.2 Effect of polymer concentration and reaction time on *E. coli* removal

The *E. coli* removal efficiency depending on the amount of polymer addition in the gel is shown in Fig. 25 (a). This result is based on 24 hours of reaction at room temperature and the concentration of hydrogel is 1% (m/v). As shown in Fig. 25 (a), a significant increase in bacterial removal effect occurred when the amount of polymer addition is increased from 0.5 mL to 0.75 mL. Also, when greater than 1.5 mL of polymer was added, the bacteria were completely removed in 24 hours. There was approximately 2-log scale of bacteria increased in the control group (control group - agarose gel only) from initial and blank group (blank - bacterial only). This increase may be caused by the components of the hydrogel. Agar is a mixture of agaropectin and agarose, which is class of polysaccharide.

(a) 24 hr reaction time



(b) Time-kill curve

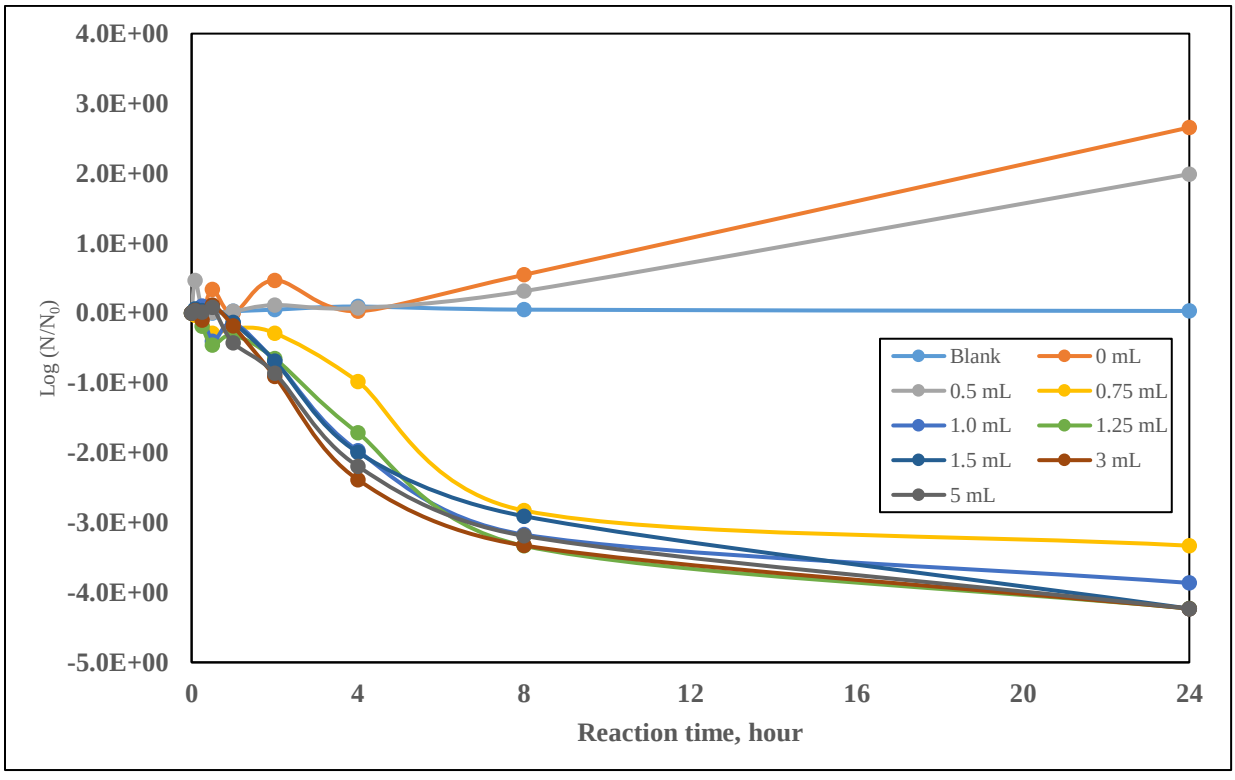


Figure 25. Effect of amount of polymer in poly-gel on *E. coli* removal.

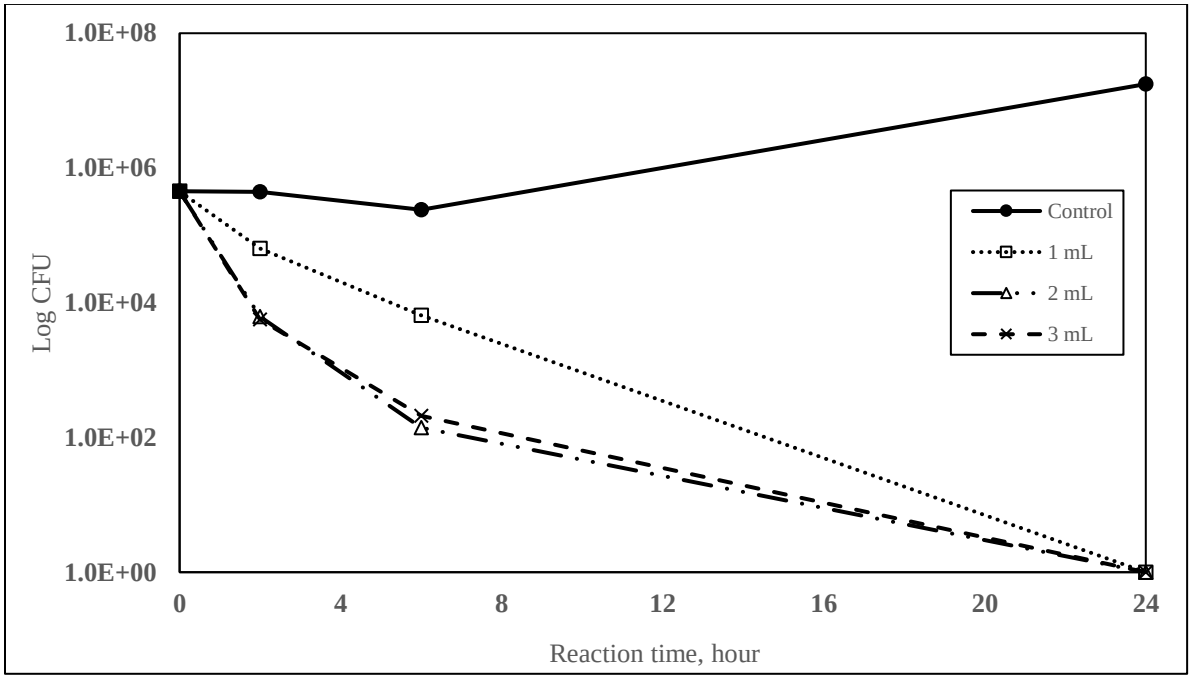
These components can act as a nutrient for bacterial growth, resulted in increase of bacterial concentration in the control group. Therefore, the bacterial removal efficiency of the polymer-gel was calculated by comparing bacterial concentration to the control, not blank. Although 0.5 mL of polymer added to the control showed a slight decrease in bacterial count compared to control, it is not considered to have removed the bacteria because it is about 2 log scale higher than blank.

The bacterial removal effect depends on the reaction time is shown in Fig. 25 (b). The higher concentration of polymer was applied, the faster the bacterial removal rate was shown. After 4 hours of reaction, significant bacterial clearance was observed compared to control, but no significant change was observed after 8 hours of reaction. The results indicated that bacterial removal by polymer-gel requires at least 4 hours of reaction time and 0.75 mL of polymer on order to see significant bacterial reduction.

V.3.3 Effect of hydrogel on *E. coli* removal

The following two hypotheses can be considered for the bacterial removal mechanism by poly-gel: 1) the polymer in the gel releases into the water and reacts with the bacteria; 2) or, adsorption of negatively charged bacterial cell is onto the gel, results in microbial inactivation in the gel. To demonstrate these hypotheses, the experiment mentioned in section 2.4.3 of Material and Methods was designed. Fig. 26 shows the effect of hydrogel on microbial removal. As shown in the Fig. 26 (a) and (b), there was a significant difference in the bacterial removal efficiency of the polymer depending on the presence or absence of hydrogel.

(a) With gel



(b) Without gel

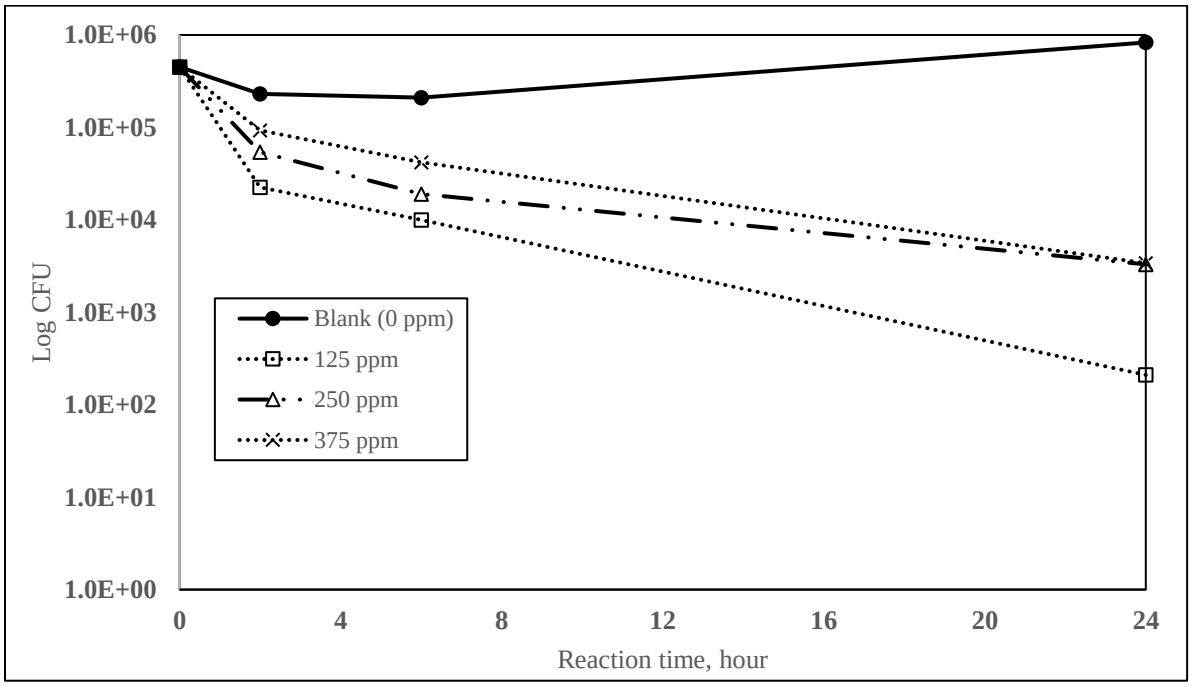


Figure 26. Physical effect of hydrogel on bacterial reduction.

(c) Poly-gel and poly-gel-liquid phase

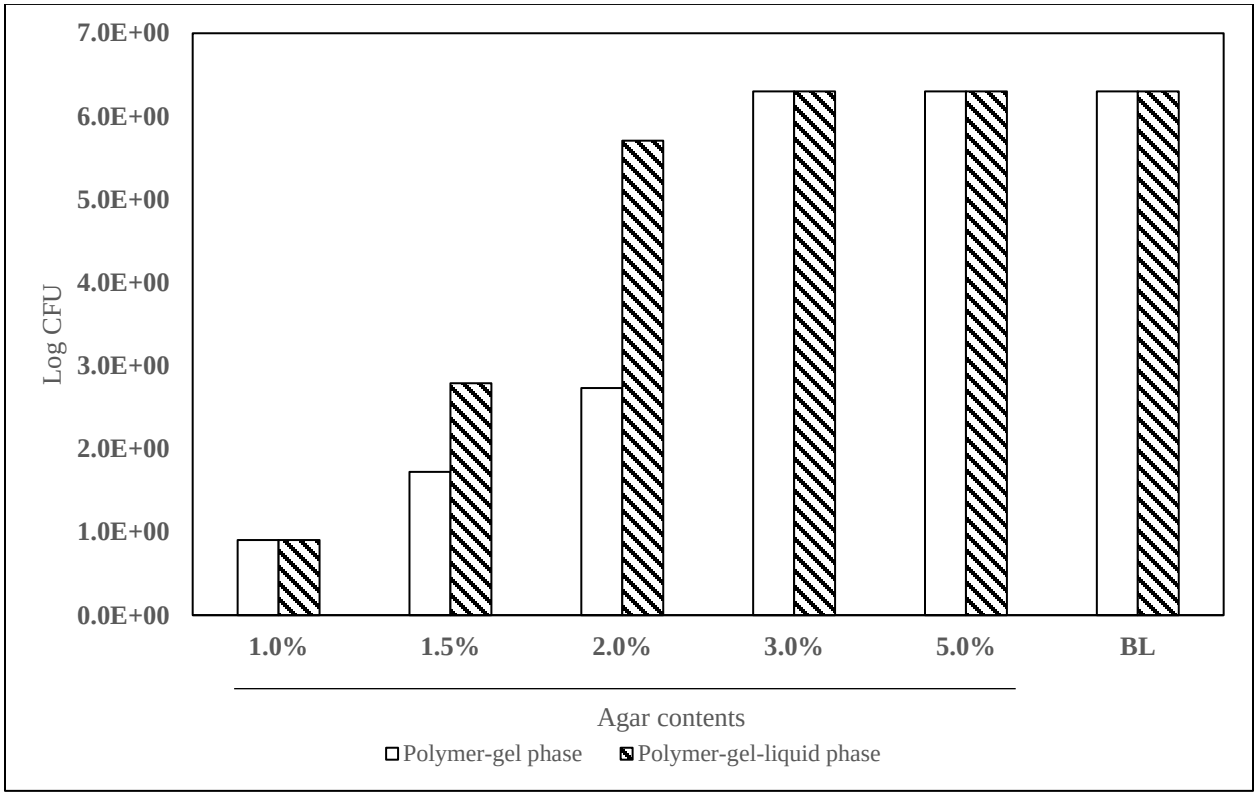


Figure 26. Continued.

In the case of polymer-gel, the significant bacterial removal was observed after 4 hours of reaction, and all the bacteria was completely removed after 24 hours, which was corresponding to the result of Fig. 25 (b).

Whereas, without gel, only 2 logs of bacterial removal were shown even at the highest concentration. These results suggest that hydrogel itself may have a great impact on the bacterial removal mechanism by polymer. At this point, we concluded that the mechanism of bacterial removal of polymer-gel is likely to be the hypothesis 2). Therefore, the effect of hydrogel

concentration on bacterial removal efficiency was conducted in order to confirm the hypothesis. Because this experiment was to confirm the effect of gel itself, the amount of polymer was fixed at 1 mL and the gel concentration was varied from 1% to 5% (w/v). The difference from the previous experiment was that 24 hours of time was given to the polymer-gel in order to allow releasing of its component into the normal saline solution. After 24 hours of extraction, half of the normal saline solution (25 mL) was collected, and same concentrations of *E. coli* was inoculated into the liquid (polymer-gel-liquid phase) and gel + liquid (polymer-gel phase) groups. Fig. 26 (c) shows the removal efficiencies of polymer-gel group and polymer-gel-liquid phase on *E. coli* depends on agar concentration.

In both polymer-gel phase and polymer-gel-liquid phases, the higher the concentration of hydrogel, the lower the bacterial removal efficiency was shown. In gels with agar concentration of 3% or more, both gel and liquid lose their ability to remove bacteria.

In 2% agar gel, the removal efficiency against *E. coli* was greater than 99.9% (3 log reduction), but the removal efficiency in the liquid phase was significantly lower compared to the gel phase. This result indicates that the physical and/or chemical properties of hydrogel play an important role in the removal of bacteria in water. As shown in the control group experiments (Fig. 26), hydrogel itself has no effect on bacterial removal, but rather it has a positive impact on bacterial viability. If so, what characteristics of the gel can affect the removal of the polymer from the bacteria? At this point I have made two more hypotheses about the role of hydrogel: 3) due to the physical properties of the gel, bacterial cells are captured in the pores of the gel to induce bacterial removal with the polymer in the gel (effect of physical property of gel); or 4) some specific components of the agar used as a hydrogel are released into water with the polymer, then these two substances work together to make a synergistic effect to inactivate the bacteria.

Hypothesis 3 could be confirmed by fluorescent microscopic analysis with DAPI staining of *E. coli* cells. After DAPI-stained bacterial cells were reacted with polymer-gel for 24 hours, the both bacteria in the gel and liquid phase were observed by a fluorescence microscopic analysis. Fluorescence microscopy showed that a certain number of bacterial cells could penetrate the gel after 24 hours of mixing. However, there was no substantial difference in the number of entrapped bacterial cells or remaining bacteria in the liquid depending on the hydrogel concentration or amount of polymer added (data not shown). Therefore, taking the results together, we concluded that the removal of bacteria by polymers and hydrogels can be attributed to the release of both components and polymers of the agar used in the hydrogel.

V.3.4 Synergistic effect of hydrogel and FL3050 against *E. coli* removal

To confirm that components of agar or agarose have a synergistic effect with the polymer, we compared the effect of agar-release-control and agar-release-polymer addition groups to remove bacteria. Fig. 27 (a), (b), and (c) showed that the higher the concentrations of agar, agarose and sucrose, the faster the bacterial removal rate. Comparison of agarose and agar showed greater removal of bacteria for agar than agarose. The reason for this result is coagulation effect when the higher the concentration of agar was applied. When higher concentration of agar was applied, aggregation of particles in the water was observed (data not shown) when the polymer was added to the liquid phase after 24-hour release time. Therefore, the higher bacterial removal in agar release can be inspired that the bacterial killing and coagulation effects are combined.

Fig. 26 (c) showed that the polymer-gel and liquid lost their ability to remove bacteria in both gel and liquid phases when higher concentration of hydrogel was applied. However, when comparing the results of Fig. 26 (c) and Fig. 27 (a), it can be deduced that the agar component

can release in water even in 3% gel because bacterial removal was occurred in the agar-release-polymer addition group. In 3% polymer-gel, it can be assumed that the polymer components or molecules could not release due to too small pore size of the hydrogel or chemical crosslinking between polymer and agar.

(a) Agar

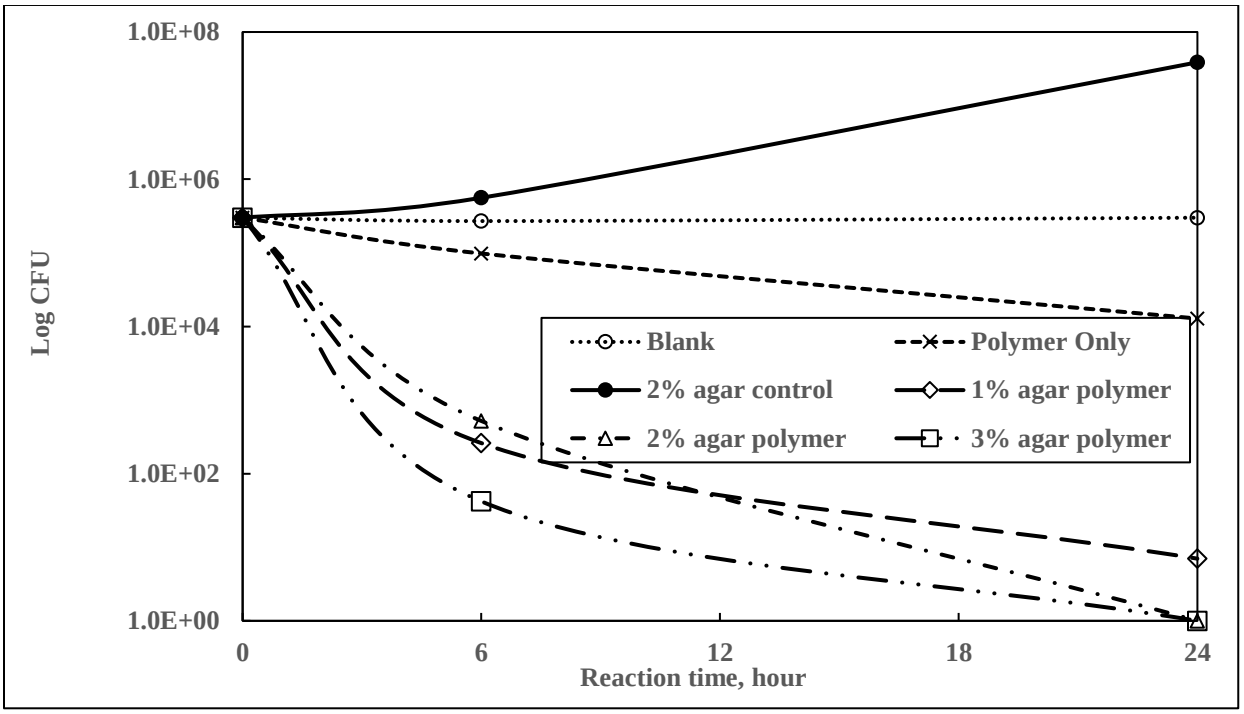
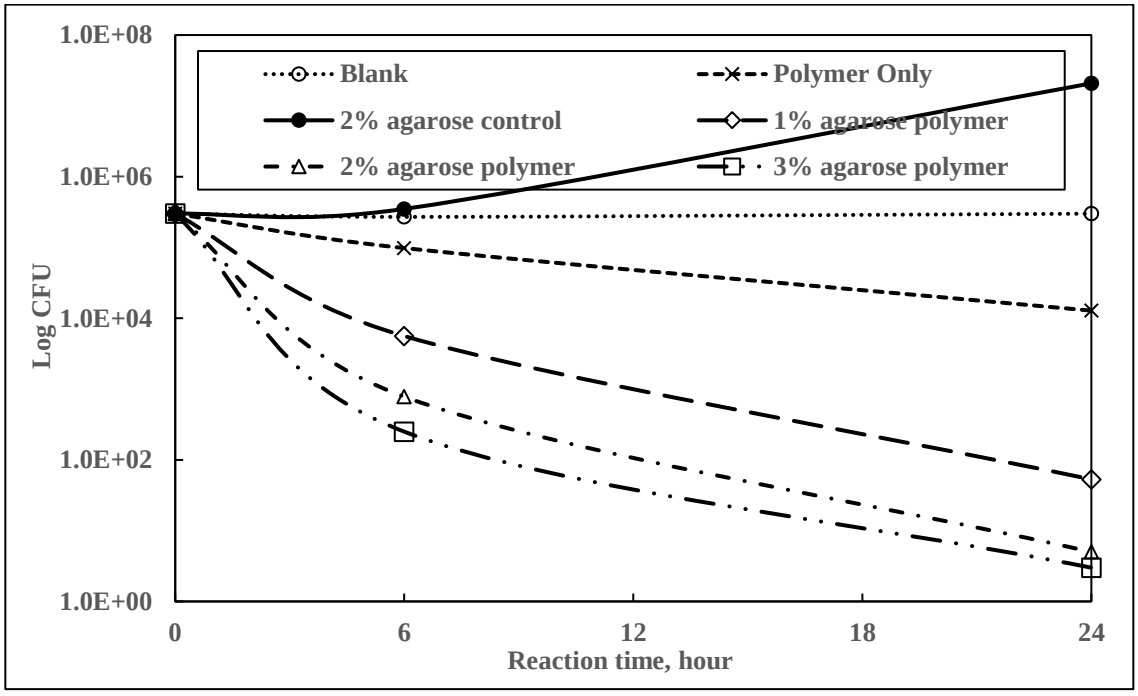


Figure 27. Chemical effect of hydrogel on bacterial reduction.

(b) Agarose



Sucrose

(c) Sucrose

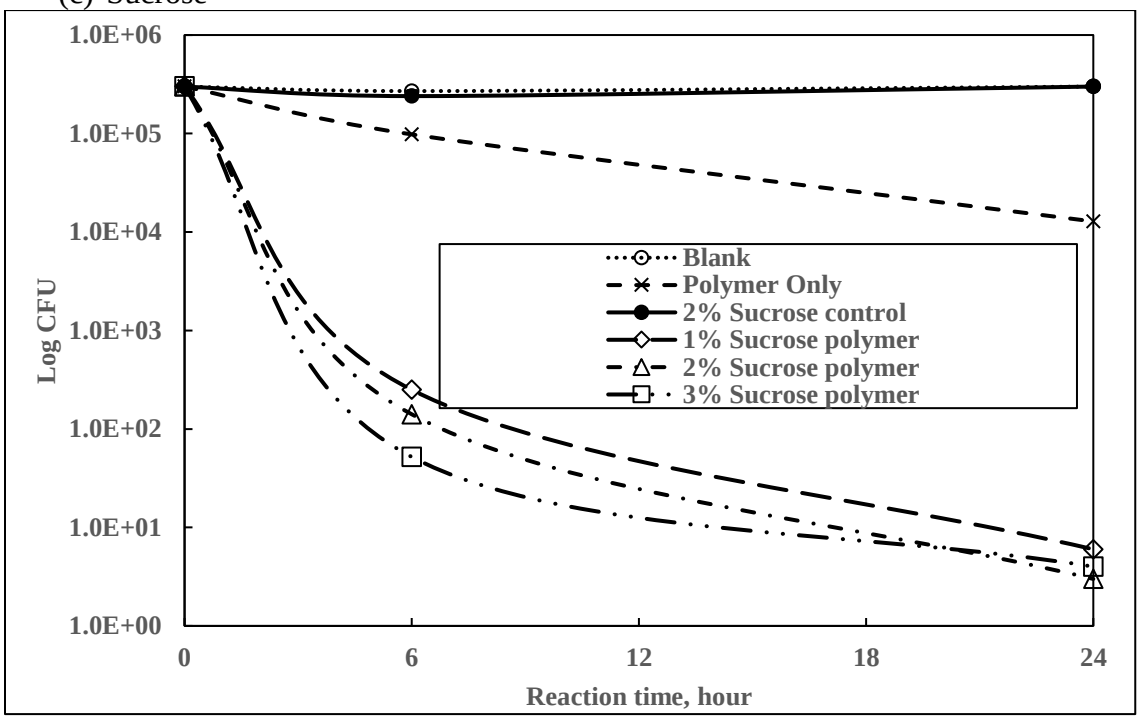


Figure 27. Continued.

Components of agar and agarose are all polysaccharides. We assumed that these polysaccharides could have a synergistic effect with the polymer in bacterial removal. To confirm the synergistic effect of sugar, sucrose was chosen to evaluate its synergistic effect. As shown in Fig. 27 (c), sucrose also has similar level of synergistic effects to other hydrogels on bacterial removal. The synergistic effect of sugar and polymer can be explained by the attraction or affinity of sugar and bacteria. Under normal conditions, bacteria are present as negatively charged particles, which not only have as an attractive force with a cationic polymer, but also a repulsion. However, sugars can more easily interact and crosslink with the polymer, and sugar can be more easily linked to bacteria. This continuous action is expected to make it easier for bacteria, including sugars, to affect the polymer.

Another possible parameter affecting polymer on bacterial removal is temperature. Fig. 26 (b) and polymer only plot of Fig. 27 indicates that the bacterial removal efficiency of 24 hours of reaction were 2 log and 1 log reduction, respectively. Although the experimental conditions of the polymer only sample are completely the same in both experiments, the reaction temperature can be considered as the possible reason for this difference. Because all of the previous experiments were conducted at room temperature except for bacterial culture for plate counting, there might be seasonal temperature differences even at a temperature-controlled laboratory. MIC and MBC result supports the difference depending on the temperature difference. As shown in Fig. 28, the MIC of the polymer treated at room temperature and 37°C shows a substantial difference of > 512 ppm and 1 ppm, respectively. Numbers on the plate in the Fig. 28 indicate the concentration of polymer in ppm (mg/L).

The synergistic effect of polymer against bacterial removal with sugar also occurred in different reaction temperature. As shown in Fig. 29, the bacterial removal ability of polyamine FL3050 was significantly decreased at 2°C, and the removal activity of the polymer was significantly increased as the reaction temperature increased. When 2% sugar and polymer were added together, the bacterial removal efficiencies were enhanced by 0.9 and 2.44 log scale at 2°C and 20°C (room temperature), respectively compared to when only polymer was added. Under high-temperature reaction conditions, synergistic effects could not be observed because all bacteria were killed for 24 hours in both the polymer group and the group containing the polymer and sugar together.

(a) Room temperature

(b) 37°C

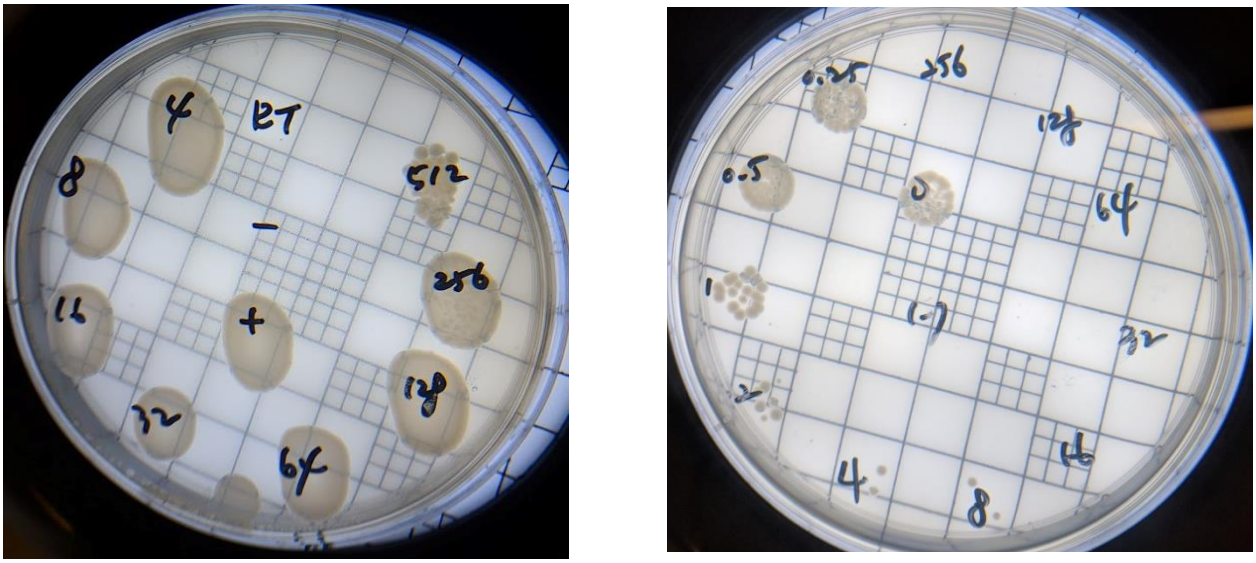


Figure 28. MIC and MBC of polymer in different temperature.

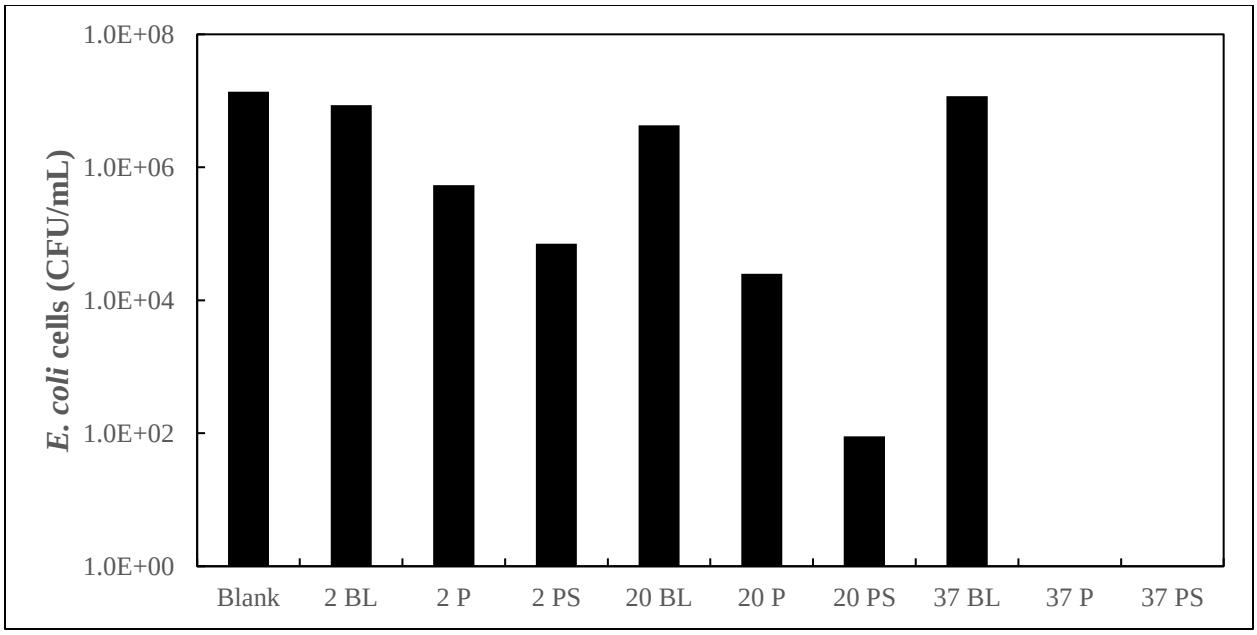


Figure 29. Synergistic effect of FL-3050 and sucrose against *E. coli* removal in different temperature. * BL, blank – bacteria only; *P*, polymer only; *PS*, polymer with sugar.

The curves in Fig. 30 shows the time dependent bacterial inactivation bacteria removal rate in different reaction temperature during 48 hours of reaction period. As shown in the Fig. 30, the sample containing sugar and polymer showed faster bacteria removal performance. Even at 37°C, although all bacteria were killed in both groups within 24 hours, the time kill results show that the combination of sugar and polymer showed a significant higher rate of bacterial inactivation. From these results, it can be confirmed that polysaccharides components such as sucrose or agarose promote the bacterial killing effect of polyamine FL3050, which is considered to be a great discovery in the agriculture, irrigation using agricultural wastewater, or water treatment industry using polymers.

(a) 2°C

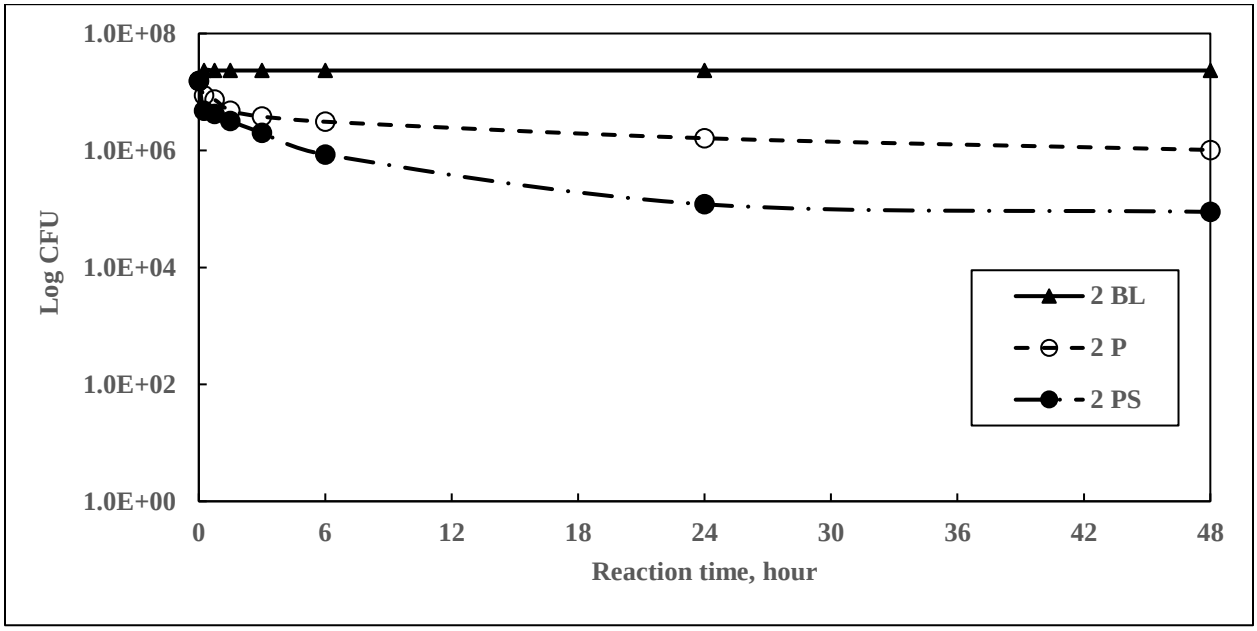
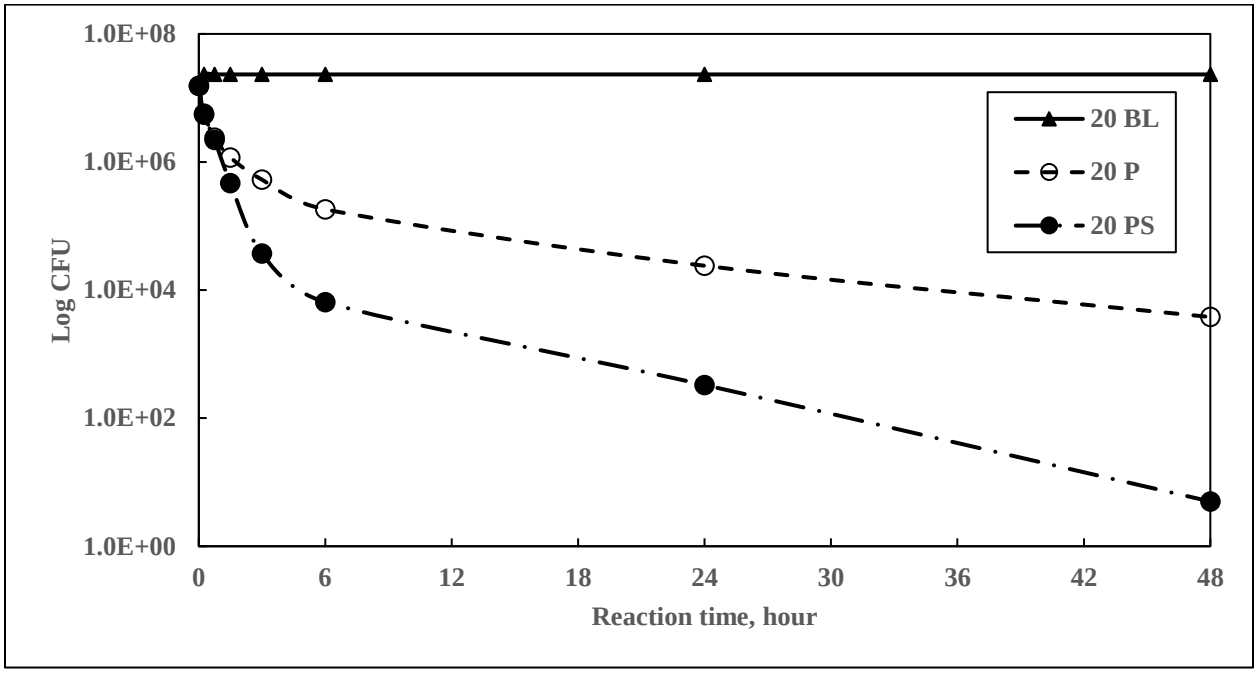


Figure 30. Time dependent synergistic effect of FL-3050 and sugar against *E. coli* removal in different temperature. (a), 2°C; (b) 20°C (room temperature); and (c) 37°C. *BL, blank – bacteria only; *P*, polymer only; *PS*, polymer with sugar.

(b) **20**°C



(c) 37°C

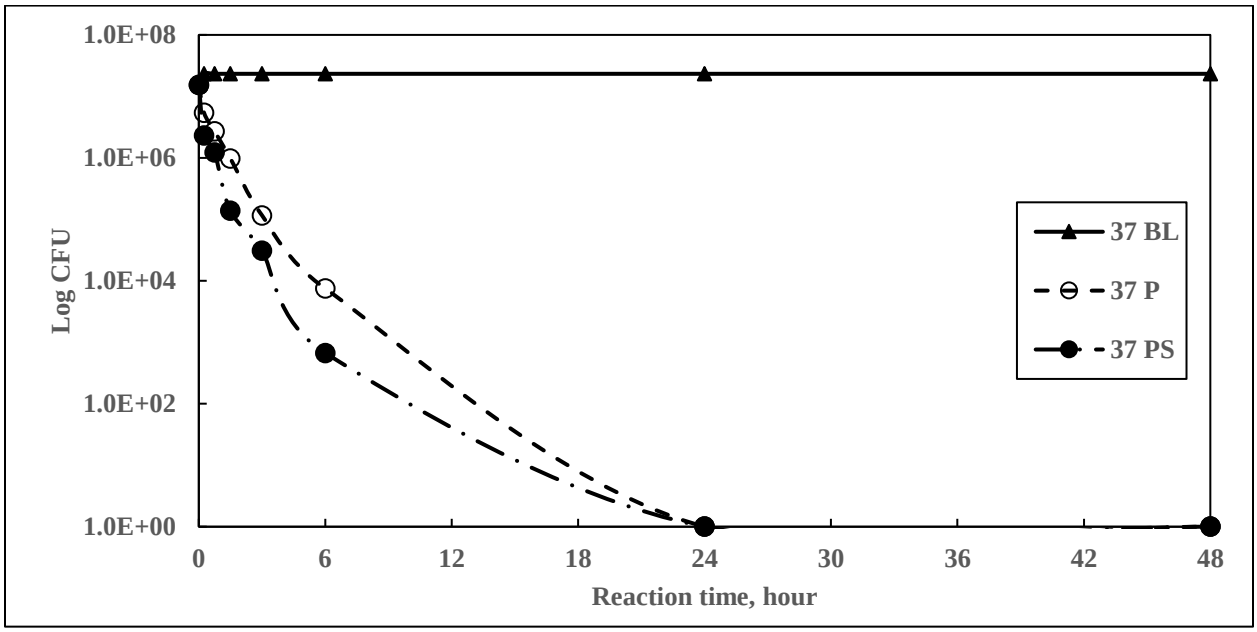


Figure 30. Continued.

V.4 Conclusion

This study suggests that using polymer with hydrogel or polysaccharides can significantly increase bacterial reduction. The following conclusions can be drawn from this study:

- Some positively charged polymer has an ability to remove bacterial in a water system.
- Bacterial removal efficiency of polymer could be enhanced when the polymer is applied with hydrogel.
- Components of hydrogel, in particular, polysaccharides have a synergistic effect on bacterial removal.
- The synergistic effects are caused by chemical properties of hydrogel, not physical properties.
- The poly-gel can be used for water treatment where microbial contamination is concerned such as manure effluent.

V.5 Summary

Polymers have been widely used as coagulant / flocculant in water treatment process. In this study, the effects of polymer and polymer containing hydrogel (poly-gel) on bacterial removal were evaluated. The types of polymer used in this study were polyacrylamide, poly-DADMAC, and polyamines. These polymers were applied by combining with hydrogels such as agar or agarose. The effect of 39 different cationic poly-gels were estimated in terms of their bacterial removal efficiency. A polymer that showed the highest bacterial removal (FL3050, polyamine) was selected as the final candidate for further study. During the experiments, it was discovered that the poly-gel exhibited remarkably higher bacterial removal performance than the polymer itself was applied. It was demonstrated that bacterial removal efficiency was more than 5 log-scale higher when the polymer was applied with hydrogel. To confirm the synergistic effect of hydrogel and polymer on bacterial removal, different experimental groups (polymer-gel, polymer-gel-liquid, and gel-release-polymer addition) were compared in terms of its bacterial removal ability. The result implies that some common components such as polysaccharides in agar and agarose have a strong synergistic effect with FL3050 on bacterial removal in water. This study focused on the bacterial removal ability of polymers, and our poly-gel strategy is not only easily applied directly to water, but also has a stronger bacterial removal effect. The results of this study suggest that the poly-gel can be applied to treat bacterial contamination in the water environment.

CHAPTER VI

SUMMARY

The research areas lie in water quality improvement, agricultural wastewater (manure and manure effluent) treatment, water microbiology, and application of novel techniques for better water quality on and around CAFOs. In particular, agricultural wastewater (lagoon effluent) has been widely used as irrigation water in neighboring farms, but there are no strict regulations regarding the water quality of this effluent. Therefore, there have not been enough studies on the quality of agricultural wastewater. In dairy operation, natural manure treatments such as lagoon system is commonly used especially in Texas. Lagoon water is rich in nutrients suitable for microorganism's growth such as nitrogen and phosphorus, there is a high risk of microbial contamination in the surrounding environment if used without proper treatment or management. The purpose of this study is to solve this problem and manure water quality, particularly focused on reducing microbial contamination.

The research is entitled "Investigation on Methods and Mechanisms of Bacteria Reduction in Agricultural Wastewater". Through two interrelated sub-studies, the research examines, first, the use of activated iron media (AIM) for reducing pathogen indicator and its bactericidal mechanism. In this study, I evaluated the effect of AIM on indicator organism reduction and its bacterial removal mechanism. AIM removed the bacterial indicator very effectively. It was confirmed that AIM removes bacteria through a combination of adsorption and inactivation in the water system. Bacterial adsorption was confirmed to be a very rapid process at the beginning of the reaction, which could be determined by fluorescence microscopy.

Bacterial inactivation was explained by the ROS reaction which was generated by AIM oxidation. Through TEM images and ROS scavenger assay, it was confirmed that ROS directly damages the cell membrane, and in particular, it was found that singlet oxygen plays a critical role in the bacterial inactivation mechanism of AIM.

Second, I examined the use of cationic polymer as a water treatment material. A novel approach in polymer application was studied, and the synergistic effect of polymer and hydrogel on bacterial removal was assessed. A total of 39 polymers from three different types (PAM, PolyDADMAC, and Polyamine) were evaluated in terms of their bacterial removal abilities, and among them, FL3050 (polyamine), which had the best bacteria removal effect, was finally selected for further research. In this study, it was confirmed that positively charged polyamine has a very significant synergistic effect when it is in combination with a polysaccharide. Also, it is confirmed that the synergistic effect is not because of the physical properties of the hydrogel, but because of a chemical synergistic effect between the gel component and polysaccharide. It is expected that the synergistic effect is due to higher affinity with the gel component and the polymer than bacterial cell and polymer. Therefore, it is strongly suggested that application of the positively charged polymer to treat bacteria in the water system, it is a very good strategy to apply polymer with hydrogel, which can reduce side effects caused by the polymer addition, and increase the bacteria removal performance.

As the quality of human life improves, the production of various types of dairy products is required, and according to the demands, the development of various types and large-scale dairy operations have been developed. However, with the creation of such a large facility resulted in an increase of the waste generated from the facility. In addition, considerations on the

treatment of waste generated from dairy farm operations such as livestock manure were not well developed and managed.

The Ph.D. dissertation research focused on the treatment of wastewater produced from animal farm, which is a very representative waste in the livestock industry, and I mainly focused on a method to reduce microbial contamination in the water system. In particular, the focus was on lagoon water treatment, the most widely used treatment method in Texas, and the target of this study can be said to be a plan to reduce microbial contamination of lagoon effluent. As a method to reduce microbial contamination, two methods using 1) AIM and 2) Cationic Polymer were investigated. The results from this study showed that both methods had excellent effects on microbial removal in the water system. In addition, research was conducted on the bacterial removal or inactivation mechanism of the applied methods against a bacterial indicator organism. Although there is a limitation in applying it to the actual field solely depends on the results of this study since this study was conducted on a lab scale, and its economic evaluation must also be demonstrated because it can be an extra step of the operation which can be incurred at an additional cost, the results obtained in this study showed very positive perspective in terms of reducing potential environmental pollution.

REFERENCES

Alekshun, M. N., and Levy, S. B. (1999) The mar regulon: multiple resistance to antibiotics and other toxic chemicals. *Trends in Microbiology*. **7**:410–413.

American Public Health Association (APHA) (2005) Standard methods for the examination of water and wastewater. Washington DC: American Water Works Association, Water Environment Federation.

Amuda, O., and Alade, A. (2006) Coagulation/flocculation process in the treatment of abattoir wastewater. *Desalination*. 196:22–31.

Angenent, L. T., Mau, M., George, U., Zahn, J. A., and Raskin, L. (2008) Effect of the presence of the antimicrobial tylosin in swine waste on anaerobic treatment. *Water Research*. 42:2377–2384.

Arikan, O. A., Mulbry, W., and Rice, C. (2009) Management of antibiotic residues from agricultural sources: Use of composting to reduce chlortetracycline residues in beef manure from treated animals. *Journal of Hazardous Materials*. 164:483–489.

Bancirova, M. (2011) Sodium azide as a specific quencher of singlet oxygen during chemiluminescent detection by luminol and Cypridina luciferin analogues. *Luminescence*. 26:685–688.

Batt, A. L., Snow, D. D., and Aga, D. S. (2006) Occurrence of sulfonamide antimicrobials in private water wells in Washington County, Idaho, USA. *Chemosphere*. 64:1963–1971.

Benjamin, M. M., and Lawler, D. F. (2013) Water quality engineering: Physical/chemical treatment processes. First edition. Wiley.

Bicudo, J. R., and Goyal, S. M. (2003) Pathogens and manure management systems: a review. *Environ Technol*. **24(1)**:115–130.

Blanco-Fernandez, B., Lopez-Viota, M., Concheiro, A., and Alvarez-Lorenzo, C. (2011) Synergistic performance of cyclodextrin-agar hydrogels for ciprofloxacin delivery and antimicrobial effect. *Carbohydrate Polymers*. 85:765–774.

Blowes, D. W., Ptacek, C. J., Benner, S. G., McRae, C. W. T., Bennett, T. A., and Puls, R. W. (2000) Treatment of inorganic contaminants using permeable reactive barriers. *Journal of Contaminant Hydrology.* 45:123–137.

Bolton, L. F., Kelly, L. C., Lee, M. D., Fedorka-Cray, P. J., and Maurer, J. J. (1999) Detection of multidrug-resistant Salmonella enterica serotype typhimurium DT104 based on a gene which confers cross-resistance to florfenicol and chloramphenicol. *Journal of Clinical Microbiology*. 37:1348–1351.

Bouki, C., Venieri, D., and Diamadopoulos, E. (2013) Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: A review. *Ecotoxicology and Environmental Safety*. 91:1–9.

Bound, J. P. and Voulvoulis, N. (2004) Pharmaceuticals in the aquatic environment-A comparison of risk assessment strategies. *Chemosphere*. 56:1143–1155.

Boxall, A. B. A., Fogg, L. A., Blackwell, P. A., Kay, P., Pemberton, E. J., and Croxford, A. (2004) Veterinary medicines in the environment. *Reviews of Environmental Contamination and Toxicology*. 180:1–91.

Btatkeu-K, B. D., Olvera-Vargas, H., Tchatchueng, J. B., Noubactep, C., and Caré, S. (2014) Determining the optimum Fe0 ratio for sustainable granular Fe0/sand water filters. *Chemical Engineering Journal*. 247:265–274.

Carmona-Ribeiro, A. M., and de Melo Carrasco, L. D. (2013) Cationic antimicrobial polymers and their assemblies. *Int J Mol Sci*. 14:9906–9946.

Chee-Sanford, J. C., Mackie, R. I., Koike, S., Krapac, I. G., Lin, Y. F., Yannarell, A. C., Maxwell, S., and Aminov, R. I. (2009) Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. *Journal of Environmental Quality*. 38:1086–1108.

Chen, J., Xiu, Z., Lowry, G. V., and Alvarez, P. J. (2011) Effect of natural organic matter on toxicity and reactivity of nano-scale zero-valent iron. *Water Research.* 45(5):1995–2001.

Cheng, D., Yuan, S., Liao, P., Zhang, P. (2016) Oxidizing impact induced by mackinawite (FeS) Nanoparticles at oxic conditions due to production of hydroxyl radicals. *Environmental Science and Technology*. 50:11646–11653.

Choi, K. J., Kim, S. G., and Kim, S. H. (2008) Removal of antibiotics by coagulation and granular activated carbon filtration. *Journal of Hazardous Materials*. 151:38–43.

Coleman, A. W. (1980) Enhanced detection of bacteria in natural environments by fluorochrome staining of DNA. *Limnology and Oceanography*. 25(5):948–951.

Corey, E. J., Mehrotra, M. M., and Khan, A. U. (1987) Water induced dismutation of superoxide anion generates singlet molecular oxygen. *Biochemical and Biophysical Research Communications*. 145:842–846.

Davenport, A. J., Oblonsky, L. J., Ryan, M. P., and Toney, M. F. (2000) The structure of the passive film that forms on iron in aqueous environments. *Journal of the Electrochemical Society*. 147:2162–2173.

Dayem, A., Hossain, M. K., Lee, S. B., Kim K., Saha, S. K., Yang, G. -M., Choi, H. Y., and Cho, S. -G. (2017) The role of reactive oxygen species (ROS) in the biological activities of metallic nanoparticles. *International Journal of Molecular Sciences*. 18:120.

Denyer, S. P. (1995) Mechanisms of action of antibacterial biocides. *Int Biodeter Biodegr*. **36**:227–245.

Diao, M. and Yao, M. (2009) Use of zero-valent iron nanoparticles in inactivating microbes. *Water Research*. 43(20):5243–5251.

Diaz-Cruz, M. S., Lopez de Alda, M. J., and Barcelo, D. (2003) Environmental behavior and analysis of veterinary and human drugs in soils, sediments and sludge. *TrAC Trends in Analytical Chemistry*. 22:340–351.

Dos Santos Coelho, F., Ardisson, J. D., Moura, F. C., Lago, R. M., Murad, E., and Fabris, J. D. (2008) Potential application of highly reactive Fe(0)/Fe3O4 composites for the reduction of Cr(VI) environmental contaminants. *Chemosphere.* 71(1):90–96.

Dubrovsky, N. M., Burow, K. R., Clark, G. M., Gronbery, J. M., Hamilton, P.A., Hitt, K.J., Mueller, D.K., Munn, M.D., Nolan, B.T., Puckett, L.J., Rupert, M.G., Short, T.M., Spahr, N.E., Sprague, L.A., and Wilber, W.G. (2010) The quality of our nation's waters- nutrients in the nation's stream and groundwater. *U.S. Geological Survey circular.* **1350**. USGS, Reston, VA.

Erdim, E., Badireddy, A. R., and Wiesner, M. R. (2015) Characterizing reactive oxygen generation and bacterial inactivation by a zerovalent iron-fullerene nano-composite device at neutral pH under UV-A illumination. *Journal of Hazardous Materials*. 283:80–88.

Esiobu, N., Armenta, L., and Ike, J. (2002) Antibiotic resistance in soil and water environments. *International Journal of Environmental Research*. 12(2): 133–144.

Freile-Pelegrin, Y. and Murano, E. (2005) Agars from three species of Gracilaria (Rhodophyta) from Yucatán Peninsula. *Bioresource Technology*. 96(3):295–302.

Fu, P. P., Xia, Q., Hwang, H. -M., Ray, P. C., and Yu, H. (2014) Mechanisms of nanotoxicity: Generation of reactive oxygen species. Jo*urnal of Food and Drug Analysis*. 22:64–75.

Garcia, M. C., Vanotti, M. B., and Szögi, A. A. (2007) Simultaneous separation of phosphorus sludge and manure solids with polymers. *Transactions of the ASABE*. 50(6):2205–2215.

Garzio-Hadzick, A., Shelton, D., Hill, R., Pachepsky, Y., Guber, A., and Rowland, R. (2010) Survival of manure-borne *E. coli* in streambed sediment: Effects of temperature and sediment properties. *Water Research*. 44(9):2753–2762.

Gavalchin, J. and Katz, S. E. (1994) The persistence of fecal-borne antibiotics in soil. *Journal of AOAC International*. 77:481–485.

Giger, W., Alder, W. A. C., Golet, E. M., Kohler, H. P. E., McArdell, C. S., Molner, E., Siegrist, H., and Suter, M. J. F. (2003) Occurrence and fate of antibiotics as trace contaminants in wastewater, sewage sludges, and surface waters. *CHIMIA International Journal for Chemistry*. 57:485–491.

Gollehon, N., Caswell, M., Ribaudo, M., Kellogg, R., Lander, C., and Leston, D. (2001) Confined animal production and manure nutrients. *Agriculture Information Bulletin* **No. 771**. USDA, NRCS, ERS.

Graham, J. P. and Nachman, K. E. (2010) Managing waste from confined feeding operations in the U.S. *J Water Health*. **8(4)**:646–670.

Gullick, R. W., Brown, R. A., and Cornwell, D. A. (2007) Source water protection for concentrated animal feeding operations: a guide for drinking water utilities. *Awwa Research Foundations and the U.S. environmental Protection Agency*.

Han, S., Huang, Y., and Liu, Z. (2019) Bacterial indicator reduction in dairy manure using hybrid zero-valent iron (h-ZVI) system. *Environmental Science and Pollution Research.* 26:10790–10799.

Helland, T., Bergheim, M., Längin, A., Kallenborn, R., and Kümmerer, K. (2010) Temperature dependent degradation of benzylpenicillin (Penicillin-G) under controlled laboratory conditions. *Chemosphere*. 81:1477–1485.

Hou, W. H. and Jafvert, C. T. (2009) Photochemistry of Aqueous C60 clusters: Evidence of $^1O_2$ formation and its role in mediating C60 phototransformation. *Environmental Science and Technology*. 43:5257–5262.

Huang, Y. and Zhang, T. C. (2005) Effects of dissolved oxygen on formation of corrosion products and concomitant oxygen and nitrate reduction in zero-valent iron systems with or without aqueous Fe^{2+}. *Water Research*. 39(9):1751–1760.

Huang, Y., Peddi, P. K., Tang, C., Zeng, H., and Teng, X. (2013) Hybrid zero-valent iron process for removing heavy metals and nitrate from flue-gas-desulfurization wastewater. *Separation and Purification Technology*. 118:690–698.

Huang, Y., Tang, C., and Zeng, H. (2012) Removing molybdate from water using a hybridized zero-valent iron/magnetite/Fe(II) treatment system. *Chemical Engineering Journal*. 200–202:257–263

Huang, Y., Zhang, T. C., Shea, P. J., and Comfort, S. D. (2003) Effects of oxide coating and selected cations on nitrate reduction by iron metal. *Journal of Environmental Quality*. 32:1306–1315.

Islam, M., Doyle, M. P., Phatak, S. C., Millner, P., and Jiang, X. (2004) Persistence of enterohemorrhagic *Eschierichai coli* O157:H7 in soil and on leaf lettuce and parsley grown in fields treated with contaminated manure composts or irrigation water. *Journal of Food Protection*. 67(7):1365–1370.

Joo, S. H., Feitz, A. J., Sedlak, D. L., and Waite, T. D. (2005) Quantification of the oxidizing capacity of nano particulate zero-valent iron. *Environmental Science and Technology*. 39:1263–1268.

Kang, S. -H. and Choi, W. (2009) Oxidative degradation of organic compounds using zero-valent iron in the presence of natural organic matter serving as an electron shuttle, *Environmental Science and Technology*. 43:878–883.

Keenan, C. R. and Sedlak, D. L. (2008) Factors affecting the yield of oxidants from the reaction of nanoparticulate zero-valent iron and oxygen. *Environmental Science and Technology.* 42:1262–1267.

Keenan, C. R. and Sedlak, D. L. (2008) Ligand-enhanced reactive oxidant generation by nanoparticulate zero-valent iron and oxygen. *Environmental Science and Technology*. 42:6936–6941.

Keenan, C. R., Goth-Goldstein, R., Lucas, D., and Sedlak, D. L. (2009) Oxidative stress induced by zero-valent iron nanoparticles and Fe (II) in human bronchial epithelial cells. *Environmental Science and Technology.* 43:4555–4560.

Kemper, N., Färber, H., Skutlarek, D., and Krieter, J. (2008) Analysis of antibiotic residues in liquid manure and leachate of dairy farms in northern Germany. *Agricultural Water Management*. 95:1288–1292.

Kenawy, E., Abdel-Hay, F., El-Shanshoury, A. E. R., and El-Newehy, M. H. (2002) Biologically active polymers. V. Synthesis and antimicrobial activity of modified poly(glycidyl methacrylate-co-2-hydroxyethylmethacrylate) derivatives with quaternary ammonium and phosphonium salts. *Journal of Polymer Science part A: Polymer Chemistry*. 40(14):2384–2393.

Kenny, J., Barber, N., Hutson, S., Linsey, K., Lovelace, J., and Maupin, M. (2009) Estimated use of water in the United States in 2005. Virginia: U.S. Geological Survey.

Keyes, K., Hudson, C., Maurer, J. J., Thayer, S., White, D. G., and Lee, M. D. (2000) Detection of Florfenicol Resistance Genes in *Escherichia coli* Isolated from Sick Chickens. *Antimicrobial Agents and Chemotherapy*. 44(2):421–424.

Khachatourians, G. G. (1998) Agricultural use of antibioitcs and the evolution and transfer of antibiotic-registant bacteria. *Can Med Assoc J*. **159**:1129–1136.

Khanna, T., Friendship, R., Dewey, C., and Weese, J. S. (2008) Methicillin resistant Staphylococcus aureus colonization in pigs and pig farmers. *Veterinary Microbiology*. 128:298–303.

Kim, E. H. and and Aoki, T. (1996) Sequence analysis of the florfenicol resistance gene encoded in the transferable R-plasmid of a fish pathogen, Pasteurella piscisida. *Microbiology and Immunology*. 40:665–669.

Kim, J. Y., Lee, C., Sedlak, D. L., Yoon, J., and Nelson, K. L. (2010) Inactivation of MS2 coliphage by Fenton's reagent. *Water Research*. 44:2647–2653.

Kim, J. Y., Park, H. J., Lee, C., Nelson, K. L., Sedlak, D. L., and Yoon J (2010) Inactivation of Escherichia coli by nanoparticulate zerovalent iron and ferrous ion. *Applied and Environmental Microbiology*. 76(22):7668–7670.

Kim, J. Y., Park, H. J., Lee, C., Nelson, K. L., Sedlak, D. L., and Yoon, J. (2010) Inactivation of *Escherichia coli* by nanoparticulate zerovalent iron and ferrous ion. *Applied Environmental Microbiology*. 76:7668–7670.

Koppenol, W. H. (1976) Reactions involving singlet oxygen and the superoxide anion. *Nature*. 262:420–421.

Kumar, K., Gupta, S. C., Chander, Y., and Singh, A. K. (2005) Antibiotic use in agriculture and its impact on the terrestrial environment. *Advances in Agronomy*. 87:1–54.

Kümmerer, K. (2009) Antibiotics in the aquatic environment – a review – Part II. *Chemosphere*. 75:435–441.

Längin, A., Alexy, R., König, A., and Kümmerer, K. (2009) Deactivation and transformation products in biodegradability testing of ß-lactams amoxicillin and piperacillin. *Chemosphere*. 75(3):347–354.

Latour, C. D. and Kolm, H. H. (1976) High-gradient magnetic separation a water-treatment alternative. *Water Research*. 68(6):325–327.

Lee, C., Keenan, C. R., and Sedlak, D. L. (2008) Polyoxometalate-enhanced oxidation of organic compounds by nanoparticulate zero-valent iron and ferrous ion in the presence of oxygen. *Environmental Science and Technology*. 42:4921–4926.

Lee, C., Kim, J. Y., Lee, W. I., Nelson, K. L., Yoon, J., Sedlak, D. L. (2008) Bactericidal effects of zero-valent iron nanoparticles on *Escherichia coli*. *Environmental Science and Technology*. 42:4927–4933.

Levy, S. B. (1992) The antibiotic paradox. How miracle drugs are destroying the miracle. Plenum Press, NY.

Li, M. Y., Cline, C. S., Koker, E. B., Carmichael, H. H., Chignell, C. F., and Bilski, P. (2001) Quenching of singlet molecular oxygen ($^1O_2$) by azide anion in solvent mixtures. *Photochemistry and Photobiology*. 74:760–764.

Liu, Z. and Wang, X. (2019) Manure Treatment and Utilization in Production Systems. Animal Agriculture 1st Edition, *Sustainability*. Challenges and Innovations. Elsevier.

Liu, Z., Carroll, Z. S., Long, S. C., Gunasekaran, S., and Runge, T. (2016) Use of cationic polymers to reduce pathogen levels during dairy manure separation. *Journal of Environmental Management*. 166:260–266.

Liu, Z., Carroll, Z. S., Long, S. C., Roa-Espinosa, A., and Runge, T. (2017) Centrifuge separation effect on bacterial indicator reduction in dairy manure. *Journal of Environmental Management*. 191:268–274.

Liu, Z., Sharara, M., Gunasekaran, S., and Runge, T. (2015) Effects of large-scale manure treatment processes on pathogen reduction, protein distributions, and nutrient concentrations. *Transactions of the ASABE*. 59:695-702.

Lorimor, J., Powers, W., and Sutton, A. (2004) Manure characteristics. MWPS-18-S1. Ames Iowa: MidWest Plan Service.

Luo, J., Song, G., Liu, J., Qian, G., and Xu, Z. P. (2014) Mechanism of enhanced nitrate reduction via micro-electrolysis at the powdered zero-valent iron/activated carbon interface. *Journal of Colloid and Interface Science*. 435:21–25.

Mackie, R. I., Koike, S., Krapac, I., Chee-Sanford, J., Maxwell, S., and Aminov, R. I. (2006) Tetracycline residues and tetracycline resistance genes in groundwater impacted by swine production facilities. *Animal Biotechnology*. 17:157–176.

Macrae, I. C. and Evans, S. K. (1983) Factors influencing the adsorption of bacteria to magnetite in water and wastewater. *Water Research*. 17(3):271–227.

Mahne, N., Schafzahl, B., Leypold, C., Leypold, M., Grumm, S., Leitgeb, A., Strohmeier, G. A., Wilkening, M., fontaine, O., Kramer, D., Slugovc, C., Borisov, S. M., and Freunberger, S. A. (2017) Singlet oxygen generation as a major cause for parasitic reactions during cycling of aprotic lithium-oxygen batteries. *Nature Energy*. 2:17036.

Martinez, J. L. (2009) Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environmental Pollution*. 157:2893–2902.

Massé, I. D., Cata-Saady, N. M., and Gilbert, Y. (2014) Potential of Biological Processes to Eliminate Antibiotics in Livestock Manure: An Overview. *Animals*. 4:146–163.

Mellon, M., Benbrook, C., and Benbrook, K. (2001) Hogging it!: Estimates of Antimicrobial Abuse in Livestock. Cambridge, MA: Union of Concerned Scientists.

Miettinen, I. T., Vartiainen, T., Martikainen, P. J. (1997) Phosphorus and bacterial growth in drinking water. *Applied Environmental Microbiology*. 63(8):3242–3245.

Morris, B. L., Lawrence, A. R. L., Chilton, P. J. C., Adams, B., Calow, R. C., and Klinck, B. A. (2003) Groundwater and its susceptibility to degradation: A global assessment of the problem and options for management. Keyworth: British Geological Survey.

Morrison, S. J., Metzler, D. R., and Dwyer, B. P. (2002) Removal of As, Mn, Se, U, V and Zn from groundwater by zero-valent iron in a passive treatment cell: reaction progress modeling. *Journal of Contaminant Hydrology*. 56:99–116.

Muñoz-Bonilla, A. and Fernández-García, M. (2012) Polymeric materials with antimicrobial activity. *Progress in Polymer Science*. 37:281–339.

National Research Council. (1999) The Use of Drugs in Food Animals: Benefits and Risks. Washington DC: National Academy Press.

Noubactep, C. (2011) On the mechanism of microbe inactivation by metallic iron. *Journal of Hazardous Materials*. 198:383–386.

Noubactep, C., Btatkeu, K. B. D., and Tchatchueng, J. B. (2011) Impact of MnO_2 on the efficiency of metallic iron for the removal of dissolved Cr^{VI}, Cu^{II}, Mo^{VI}, Sb^{V}, U^{VI} and Zn^{II}. *Chemical Engineering Journal*. 178:78–84.

Pathak, S. H. C. (2010) Antibiotics in Manure and Soil-A Grave Threat to Human and Animal Health. New Delhi: National Academy of Agriculture Science.

Payne, J. B. and Lawrence, J. (2015) Manure as a Source of Crop Nutrients and Soil Amendment. Cooperative Extension. https://articles.extension.org/pages/8913/manure-as-a-source-of-crop-nutrients-and-soil-amendment. Accessed 26 October 2015.

Qiang, Z. H., and Adams, C. (2004) Potentiometric determination of acid dissociation constants (pKa) for human and veterinary antibiotics. *Water Research*. 38:2874–2890.

Ray, P. D., Huang, B. -W., and Tsuji, Y. (2012) Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell Signaling Technology*. 24:981–990.

Redman, A. D., Macalady, D. L., and Ahmann, D. (2002) Natural organic matter affects arsenic speciation and sorption onto hematite. *Environmental Science and Technology*. 36:2889–2896.

Ribaudo, M., Gollehon, N., Aillery, M., Kaplan, J., Johansson, R., Agapoff, J., Christensen, L., Breneman, V., and Peters, M. (2003) Manure management for water quality costs to

animal feeding operations of applying manure nutrients to land. USDA-ERS Agricultural Economic Report 824.

Rijnaarts, H. H. M., Norde, W., Lyklema, J., and Zehnder, A. J. B. (1994) The isoelectric point of bacteria as an indicator for the presence of cell surface polymers that inhibit adhesion. *Colloids and Surfaces B: Biointerfaces*. 4:191–197.

Rodriguez, G. G., Phipps, D., Ishiguro, K., and Ridgway, H. F. (1992) Use of fluorescent redox probe for direct visualization of actively respiring bacteria. *Applied Environmental Microbiology*. 58(6):1801–1808.

Rogers, S. W., Donnelly, M., Peed, L., Mondal, S., Zhang, Z., and Shanks, O. (2011) Decay of bacterial pathogens, fecal indicators, and real time quantitative PCR genetic markers in manure-amended soils. *Appl Environ. Microb*. **77(14)**:4839–4848.

Saito, H. and Nosaka, Y. (2014) Mechanism of singlet oxygen generation in visible-light-induced photocatalysis of gold-nanoparticle-deposited titanium dioxide. *The Journal of Physical Chemistry C: Nanomaterials and Interfaces.* 118:15656–15663.

Salton, M. R. J. (1968) Lytic agents, cell permeability and monolayer penetrability. *J Gen Physiol*. **52**:277S–252S.

Salyers, A. A., Gupta, A., and Wang, Y. (2004) Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends in Microbiology*. 12:412–416.

Sarmah, A. K., Meyer, M. T., and Boxall, A. B. A (2006) A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere*. 65(5):725–759.

Scherer, M. M., Richter, S., Valentine, R. L., and Alvarez, P. J. (2000) Chemistry and microbiology of permeable reactive barriers for in situ groundwater clean up. *Critical Reviews in Microbiology.* 26(4):221–264.

Shea, K. M. (2003) Antibiotic resistance: What is the impact of agricultural uses of antibiotics on children's health? *Pediatrics*. 112:253–258.

Shi, J., Ai, Z., and Zhang, L. (2014) Fe@Fe2O3 core-shell nanowires enhanced Fenton oxidation by accelerating the Fe(III)/Fe(II) cycles. *Water Research*. 59:145–153.

Shokes, T. E. and Moller, G. (2000) Removal of dissolved heavy metals from acid rock drainage using iron metal. *Environmental Science and Technology*. 33:282–287.

Singer, A. C., Shaw, H., Rhodes, V., and Hart, A. (2016) Review of antimicrobial resistance in the environment and its relevance to environmental regulators. *Frontiers in Microbiology*. 7:1728.

Smith, T. C., Gebreyes, W. A., Abley, M. J., Harper, A. L., Forshey, B. M., Male, M. J., Martin, H. W., Molla, B. Z., Sreevatsan, S., Thakur, S., Thiruvengadam, M., and Davies, P. R. (2013) Methicillin-resistant *Staphylococcus aureus* in pigs and farm workers on conventional and antibiotic-free swine farms in the USA. *Plos One*. 8(5):63704.

Solomons, I. A. (1978) Antibioitcs in animal feeds – Human and animal safety issues. *J Ani Sci*. **46**:1360–1368.

Szögi, A. A., Vanotti, M. B., and Hunt, P. G. (2006) Dewatering of phosphorus extracted from liquid swine waste. *Bioresource Technology*. 97(1):183–190.

Tang, C., Huang, Y., Zhang, Z., Chen, J., Zeng, H., and Huang, Y. (2016) Rapid removal of selenate in a zero-valent iron/Fe3O4 /Fe2+ synergetic system. *Applied Catalysis B: Environmental*. 184:320–327.

Tapias, G. N., Sicchierolli, S. M., Mamizuka, E. M., and Carmona-Ribeiro, A. M. (1994) Interactions between cationic vesicles and *Escherichia coli*. *Langmuir*. **10**:3461–3465.

Timothy, F. L., Cohen, B., Wittum, T. E., and Larson, E. L. (2012) A review of antibiotic use in food animals: Perspective, policy, and potential. *Public Health Rep*. **127(1)**: 4–22.

Tombácz, E., Majzik, A., Horvát, Z., and Illés, E. (2006) Magnetite in aqueous medium: Coating its surface and surface coated with it. *Romanian Reports in Physics*. 58(3):281–286.

Touati, D. (2000) Iron and oxidative stress in bacteria. *Archives of Biochemistry and Biophysics*. 373(1):1–6.

United States Environmental Protection Agency (2013) Literature review of contaminants in livestock and poultry manure and implications for water quality. Office of Water. EPA 820-R-13-002.

Vanotti, M. B., Rashash, D. M. C., and Hunt, P. G. (2002) Solid-Liquid separation of flushed swine manure with PAM: Effect of wastewater strength. *Transactions of the ASAE*. 45(6):1959–1969.

Vanotti, M., Rashash, D., and Hunt, P. (2002) Solid-liquid separation of flushed swine manure with PAM: effect of wastewater strength. *Transactions of the ASAE*. 45:1959–1970.

Williams, P. W. and Phillips, G. O. (2000) "Chapter 2: Agar". Handbook of hydrocolloids. Cambridge: Woodhead. P.91. ISBN 1–85573–501–6.

Witte, W. (2000) Selective pressure by antibiotic use in livestock. *Int J Antimicrob Agents*. **16**:S19–S24.

Wong, S., Teng, T., Ahmad, A., Zuhairi, A., and Najafpour, G. (2006) Treatment of pulp and paper mill wastewater by polyacrylamide (PAM) in polymer induced flocculation. *Journal of Hazardous Materials*. 135:378–388.

Wu, H., Yin, J. -Jie., Wamer, W. G., Zeng, M., and Lo, Y. M. (2014) Reactive oxygen species-related activities of nano-iron metal and nano-iron oxides. *Journal of Food and Drug Analysis*. 22:86–94.

Xu, J., Tang, J., Baig, S. A., Lv, X., and Xu, X. (2013) Enhanced dechlorination of 2,4-dichlorophenol by Pd/FeFe3O4 nanocomposites. *Journal of Hazardous Materials*. 244–245:628–636.

Yesilgul, N., Uyar, T. B., Seven, O., and Akkaya, E. U. (2017) Singlet oxygen generation with chemical excitation of an erythosine luminol conjugate. *ACS Omega*. 2:1367–1371.

Zhang, R. and Westerman, P. (1997) Solid-liquid separation of animal manure for odor control and nutrient management. *Applied Engineering in Agriculture*. 13:385–393.

APPENDIX I

16S rRNA sequence information of isolated bacterial strain

TGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATGAGC

AAAGGTATTAACTTTACTCCCTTCCTCCCCGCTGAAAGTACTTTACAACC

CGAAGGCCTTCTTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCAT

TGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTC

AGTTCCAGTGTGGCTGGTCATCCTCTCAGACCAGCTAGGGATCGTCGCCT

AGGTGAGCCGTTACCCCACCTACWAGCTAATCCCATCTGGGCACATCCGA

TGGCAAGAGGCCCGAAGGTCCCCCTCTTTGGTCTTGCGACGTTATGCGGT

ATTAGCTACCGTTTCCAGTAGTTATCCCCCTCCATCAGGCAGTTTCCCAG

ACATTACTCACCCGTCCGCCACTCGTCAGCGAA