

**LOW COST SOIL-BASED BIOLOGICAL TREATMENT
FOR WATER RECLAMATION**

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ABSTRACT

Water reclamation is a strategy of moving toward sustainable management of freshwater and environmental protection. Treated wastewater has been widely recognized as a potential source of water for landscape and agricultural irrigation, industrial cooling, surface replenishment, groundwater recharge, portable and non-portable use for the past decades. However, concerns about pathogenic organisms and trace organic contaminants in reclaimed water remain. Low-cost treatment methods show promise in reducing these contaminants in wastewater, but more investigation of these technologies is still needed to improve the efficiency for renewable and sustainable water reclamation.

This study presents the soil based filter as a treatment means to remove bacteria in agricultural and domestic effluents for water reclamation. An improved soil filter by ferric oxide based materials integrated with native soil protozoa bacterivory was efficient to eliminate *E. coli* in swine wastewater. Under anaerobic environment, the microbial iron reduction (MIR) process was very efficient in inactivating *E. coli* cells. The ferrous production in MIR process was identified as a mechanism for *E. coli* inactivation under the anaerobic condition. Inactivated bacterial cells were used by the MIR community as an electron donor to drive the MIR process. The anaerobic-aerobic two-stage slow sand filter was a robust system for water reclamation. High removal efficiencies of carbon substrates, trace organic compounds, and microbial contaminants were obtained in this study. The iron oxide coated sand and MIR biofilm provided adsorptive surfaces to retain bacterial cells passing through the filter media. The integration of anaerobic iron coated sand filter and aerobic sand filtration removed not only ferrous production but also improved the overall performance of the treatment system in removing bacteria.

This dissertation has shown that the filters packed by iron-rich porous media provided technical and economic feasibilities to remove microbial contaminants in water reclamation. This knowledge could further improve our understanding of the fate and transport of fecal bacteria in the subsurface and sedimentary environments. Future work can be explored for the removal of pathogens enhanced by the mechanisms discovered in this study in engineering processes, such as storm water bio-retention facilities, aquifer artificial recharge, and low-cost soil based water reclamation.

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LIST OF ABBREVIATIONS

SSF:	Slow sand filter
HRT:	Hydraulic retention time
AMIR:	Anaerobically microbial iron reduction
MSL:	Multi-Soil-Layer
Fe ₂ O ₃ :	Ferric oxide
PEP:	Pre-enrichment protozoa
NPG:	Natural protozoa growth
CT:	Cycloheximide treated
FeOOH:	Ferric oxyhydroxide
Fe ³⁺ :	Ferric ion
Fe ²⁺ :	Ferrous ion
IRB:	Iron reducing bacteria
MIR:	Microbial iron reduction
MSR:	Microbial sulfate reduction
MNR:	Microbial nitrate reduction
GFP:	Green fluorescent protein
CLSM:	Confocal Laser Scanning Microscopy
DHA:	Dehydrogenase assay
FDA:	Fluorescein diacetate hydrolytic activity
TPF:	Triphenylformazan
CECs:	Contaminants of emerging concerns
TOC:	Total organic carbon

DOCs:	Dissolved organic carbons
WWTPs:	Waste water treatment plants
BTC:	Breakthrough curve
E1:	Estrone
E2:	17 β -Estradiol
E3:	Estriol
CBZ:	Carbamazepine
GFZ:	Gemfibrozil

CHAPTER 1. INTRODUCTION

1.1 Overview

Across the globe, freshwater sources are limited while water demand has been greatly increasing for food production, domestic use, and industries. Currently, several regions around the world are suffering from water scarcity. According to a recent report by the United Nations, seven million people are living at the lower minimum requirement for basic water needs (UNDP 2006). By 2025, approximate two billion people will be living in states or areas facing water scarcity, and a haft of the world population may be living under water stress condition (FAO 2007). Chronically, water conflict raised between water users took place in some regions around the world because of lacking freshwater source accessibility. In the United States, water conflict happened in California between the city and agricultural areas. The military tension in Middle East is relevant to the water conflict. Thus, it is utmost important to take care of the water resources by sustainable use and management of existing water resources.

1.2 Water reclamation

Wastewater is increasingly recognized as a valuable water source if it is adequately treated to ensure the water quality for appropriate uses. Landscape and agricultural irrigation, industrial use, surface water replenishment, groundwater recharge, and other portable and non-portable uses have been identified as primary applications of recycled water with suitable treatment [1]. In Singapore, domestic wastewater was successfully purified to the level that meets the drinking water standard. Although a high level of water purification was achieved after treatment, the produced water is still not

directly used for drinking but to replenish to surface water resources [2]. In the United States, treated wastewater has been extensively reused for agricultural and landscape irrigation, toilet flushing, industrial cooling, and groundwater recharge in 10 regions corresponding to the EPA [3]. Reuse of treated wastewater in Hawai'i has been employed by the county of Honolulu for over ten years as a strategy to deal with increasing water demand in the state. Oahu's Honouliuli Water Recycling Facility is the largest plant that can produce 12 million gallons per day with two types of recycled water [4]. Onsite treatment of wastewater was also investigated to reuse treated water in isolated residential areas [5]. Although water reclamation has the potential of becoming an important water source to meet the increasing of water demand, concerns about potential health risk associated with pathogenic organisms including bacteria, parasites, and viruses, as well as micropollutants still largely limit the application of reclaimed water. Therefore, , removing health risk drivers from reclaimed water before being used for appropriate purposes is essential to public health protection.

1.3 Low-cost technologies for water reclamation

Conventional wastewater treatment technologies that combine physical, chemical, and biological processes are efficient in removing suspended solids, organic matters, nutrients, and pathogens. Different treatment levels and types of technologies are more or less effective in removing the different types of contaminants (Table 1). A tertiary treatment is included in wastewater reclamation depending application purposes. In general, filtrations or disinfection are often used as the final process to remove pathogens. A recent study revealed that some levels of microbial pathogens were still present in reclaimed water in Southern Region of America after disinfection by chlorination [6],

which increased health risks when the water was used in vegetable irrigation [7]. It was also pointed out that the removal efficiency of microbial contaminants using conventional biological treatment, flocculation, sand filtration, and chlorination did not meet the criterion for land application [8].

Moreover, antibiotic resistant bacteria and genes pose the greatest potential risk to public health [9]. In addition, minimizing usage of oxidative chemicals in removing pathogens in recycled water for horticultural application has attracted attention worldwide [10]. Overuse of these chemicals can lead to the formation of disinfection byproducts (DBPs) due to oxidation of halogenated compounds by chlorine [11-13]. Advanced membrane technology was successfully implemented to eliminate contaminants from domestic wastewater to generate safe drinking water [2]. Although previous studies on nano-filtration (NF), reverse osmosis (RO), advanced oxidation, activated carbon filtration pointed out that these technologies are efficient in removing micro-pollutants and microorganisms from water [14-16], the cost of these treatment methods remains relatively high [12].

Low-cost technologies are an attractive option for water reclamation in the context of efficacy, cost-effectiveness, and sustainability. The treatment processes are based on natural factors such as activities of indigenous microorganisms and physical and chemical reactions in natural or minimally engineered environments. In comparison to conventional wastewater treatment processes, at present, low-cost technologies only account for a small percentage of the treatment landscape. However, they promise a robust, reliable and long-term efficient treatment option for water reclamation.

Table 1.1. Log removal of microbial contaminant in different treatment process *

Treatment	<i>E. coli</i>	<i>Clostridium</i>	Phage	Enteric bacteria	Enteric virus	Giardia	Crypt. parvum	Helminths
Secondary	1 - 3	0.5 - 1	0.5 - 2.5	1 - 3	0.5 - 2	0.5 - 1.5	0.5 - 1	0 - 2
Dual media filtration	0 - 1	0 - 1	1 - 4	0 - 1	0.5 - 3	1 - 3	1.5 - 2.5	2 - 3
Membrane filtration	4 - 6	>6	2 - 6	6	2 - 6	>6	4 - 6	>6
Ponds	1 - 5	-	1 - 4	1 - 5	1 - 4	3 - 4	1 - 3.5	1.5 - 3
Ozonation	2 - 6	0 - 0.5	2 - 6	2 - 6	3 - 6	2 - 4	1 - 2	-
UV	2 - 6	-	3 - 6	2 - 6	1 - 6	3 - 6	3 - 6	-
Advanced oxidation	>6	-	>6	>6	>6	>6	>6	-
Chlorination	2 - 6	1 - 2	0 - 2.5	2 - 6	1 - 3	0.5 - 1.5	0 - 0.5	0 - 1

* Adapted from Reference [3, 17]

Table 1.2. Types of treatment, appropriate uses, health risk, and cost *

Treatment levels	Primary	Secondary	Filtration and disinfection	Advanced oxidation
Process	Sedimentation	Biological and disinfection	Chemical coagulation, biological/chemical nutrient removal, filtration and disinfection.	Activated carbon, reverse osmosis, advanced oxidation processes.
Use	Not recommended for use	Irrigation, groundwater of non-potable aquifer, wetland and wildlife augmentation, industrial cooling.	Irrigation, toilet flushing, commercial and industrial uses, recreational water.	Indirect potable reuse: replenishment to groundwater, surface water, and potable reuse
Human exposure	Decreasing the health risk			
Cost	Increasing levels of treatment cost			

* Adapted from Reference [3]

1.3.1 Natural systems

a. Facultative lagoons

Facultative lagoons are a typical aquatic treatment system. The natural establishment of environmental conditions including aerobic at the top, anoxic at the middle, and anaerobic at the bottom, supports growth of various types of microorganisms at different water depths [3]. Removal of pathogens in facultative lagoons is primarily caused by predation and solar inactivation mechanisms.

b. Wetland

Wetland treatment technology is a naturally based system used to remove bacteria in wastewater for irrigation [18]. It has been used for more than 40 years, and hundreds of treatment units are still being operated in the United States [3]. Wetlands can be either constructed or used in natural land systems. In both categories, water is always maintained in soil surface with the related growth of vegetation. Swamps are natural wetlands that are transitional areas between aquatic and terrestrial ecosystems. Constructed wetland has human involvement in the design, construction, and operation, and possesses similar components to natural wetlands. The treatment types of constructed wetlands are well established by water flow that is surface or subsurface flow. According to EPA (2012), wetlands are very efficient in removing suspended solids, nitrogenous compounds, phosphorus, sulfate, and toxic substances [3]. Sedimentation, biodegradation, adsorption, plant uptake, predations are primary mechanisms removing suspended solids, organic matters, nutrients, and pathogens in the wetland systems.

c. Soil aquifer filtration

Soil filtration and percolation are preliminary treatment methods to remove contaminants from wastewater. This onsite treatment has a long history in the treatment of agricultural waste and household scale of domestic wastewater. A traditional way of using soil filtration as a means of wastewater treatment is the spreading of wastewater onto soil surface. This system requires unconfined aquifer, and soil media must have a high porosity to allow sufficient water infiltration. Although this method can be beneficial to the simultaneous removal of contaminants and recharge of groundwater, this practice has raised the concern about possible adverse effects of bacterial transport to groundwater. In 1967, an experimental soil filtration system was tested to observe the transport and fate of bacteria and virus in soil media where treated wastewater was flooded [19]. The importance of soil filtration became widely recognized in 1970. Several studies found that soil filtration is an efficient treatment method to remove organic matters, nutrients, bacteria, and viruses from treated wastewater [19-24]. The removal mechanisms depend upon physical, chemical, and biological reactions in the soil media.

1.3.2 Slow sand filtration

Slow sand filtration (SSF) is one of the oldest drinking water treatment systems. In recent years, not only is it used to purify water but it is also implemented as a tertiary treatment unit for water reclamation [10, 25-28]. SSF is recognized as a cost-effective means for treating water because the natural, cheap, and renewable packing media is used for construction, and low energy is required for operation. This treatment method has been used from the early 1800's in Europe [27]. SSF technology spread to North America in the 20th century and about 100 slow sand filtration systems were constructed by 1940

[29]. However, the SSF was reluctantly accepted in the U.S. until the late 1970's and early 1980's due to a potential removal of bacteria and virus as well as *Giardia* cysts. As a matter of fact, several SSF systems were constructed in the USA during this period (Table A.1). SSF has been widely used as a treatment means for various water applications, including wastewater, in remote areas and developing countries. Due to its simple configuration and low cost in operation and maintenance, SSF becomes more attractive in water and wastewater treatment. According to the World Health Organization (WHO), SSF can produce high quality of treated water at the lowest cost under suitable circumstance [27].

Slow sand filtration uses small diameter sand granules (0.15-0.35 mm) as filtration media [27]. The system is operated at low surface loading rate ($0.1-0.2 \text{ m}^3/\text{m}^2\text{h}$) without requiring any use of a chemical coagulant [29]. Particulates as well as suspended solids are removed in SSF systems. Straining and adsorption are two the primary mechanisms responsible for removal processes of these particles within packing media [30, 31]. When particles reside onto the spaces between the sand grains, straining phenomena will take place due to size reduction of pore spaces. SSF initiates the top surface of filtration media and expands to lower depth at the maturation stage. Physical adsorption of particles to surfaces of sand media is attributable to the combination of electrostatic interaction, van der Waals force, and adherence [27].

Although physical and biological mechanisms concurrently remove contaminants in SSF, biological mechanisms are the dominant force in removing small particles and microorganisms [30]. Significant reductions of microbial contaminants from surface water, groundwater, as well as treated wastewater effluent, have been observed in SSF

treatment methods [25, 30, 32, 33]. The biolayer called “Schmutzdecke” develops on the top media surface during operation. Schmutzdecke contains organic materials, algae, and a diversity of microorganisms. It was discovered that this layer served as a bioactive membrane purification, removing bacteria under both physical adsorption and predation [27, 30]. Previous work addressed that vigorous populations of indigenous microorganisms colonize in the top portion of SSF, and gradually decrease with depth because of food limitations [10, 27, 34, 35]. In contrast, biomass development within filtration media was not significant at different depths in covered SSF [36]. The greatest level of purifying bacteria was found in the depth of 30-40cm. Below this depth, biochemical reactions mostly took place according to the micro-environmental establishment and the availability of appropriate physico-chemical properties throughout the filter [27, 29]. Microbial growth in lower depth of filtration systems facilitates physical, biochemical adsorption and degradation of dissolved organic and microbial products released from biological processes of heterotrophic microbes at the top media surfaces.

Different species of microorganisms are found at various depths of SSF after a certain operational time. Protozoa, nematodes, flagellates, and *Xanthus* bacteria were the most detected microorganisms in the upper part of filter (5.0 cm) while ciliates, chemolithotrophs, and Proteobacteria were found to be predominant in lower depth [32, 33, 37]. The presence of protozoa and flagellates in SSF was correlated with some levels of *E. coli* removal at maturation stage [30, 32]. However, the reduction of viruses passing through SSF was most likely contributed to by activities of proteolytic bacteria and chemolithotrophs [33]. Although significant information is well known for the

contribution of activities of indigenous organisms on SSF performance, uncertain evidence has been found for manipulated establishment of a microbial community that is active against pathogenic bacteria. This lack of information limits further optimization and application of SSF in water reclamation.

1.4 Modified media surfaces in sand filtration

An update to sand filtration was recently investigated to improve the removal efficiency of biological contaminants. Surface modification achieved by coating iron oxides to supporting sand media attracts the attention of research interests in water and wastewater treatment. At a neutral pH, surfaces of iron oxide coated filtration media expose positive charge. It was reported that mineral oxides coating to media surfaces increase removal efficiencies of microorganisms [38-40]. Mineral oxides used to cover the surface of sand grains increase the capture of negative charge particulates in waters. For example, microorganisms exhibit the negative charge in wastewater and readily attach the mineral oxides that carry a positive charge at neutral pH due to electrostatic interactions. Besides the physical adsorption mechanism, this medium type may catalyze chemical reactions and microbial activities that can cause oxidative stress and inactivation of pathogenic bacteria found in water and wastewater. Mineral oxide coated sand promises to be a potential filtration media because it is inexpensive, nontoxic, and can be readily prepared [41].

Ferric oxyhydroxide coating porous media has shown to be effective in removing microorganisms in both field and laboratory experiments [38, 42-45]. Increasing absorption of protozoa cysts to porous media has a correlation to increasing fractions of

iron oxyhydroxide coating granules [46]. Other research showed that sand filtration amended with zero-valent particles or after being coated with ferric-aluminum hydroxide had more advantage in removing bacteria and virus in feeding water than that with only sand use [38, 39]. Another effort has been made to use mineral oxides in removing heavy metals from groundwater. Some work has shown that there was a high absorption affinity of heavy metals from the groundwater to iron coated sand surfaces [41, 47-49]. High immobilization rates of arsenic were observed in both field and laboratory filtration using the composite iron matrix as media [48]. Iron coated sand also served as a potential absorbent of synthetic and natural organic compounds [49-51].

Physicochemical and thermodynamic adsorptions were initially hypothesized to account for removal or immobilization of biotic and abiotic contaminants in metallic oxides coating media filtration [38, 45]. Inactivation of bacteria and virus that captured to mineral surfaces had also been observed in column studies and field experiments [43, 52, 53]. Although a recent work has shown that activities of microorganisms were increased within porous media coated with mineral oxides [54], little information is known about the contribution of biological mechanisms to bacteria removal from iron oxide coated sand filtration. Microbial reduction of ferric iron has also been shown to have positive influences in the immobilization of arsenic in groundwater [55, 56]. However, it is not well understood in a continuous flow filtration column, even though, the old fashion of SSF systems possess highly biological activities during the operation. Such a knowledge gap in iron coated sand filtration to remove microbial contaminants may hamper the advancement of surface modification SSF in optimization and practical applications in water and wastewater treatment.

The use of iron oxides coated sand for filtering media in slow sand filtration can potentially provide a robustly low-cost technology in water reclamation. Not only is iron oxide coated sand inexpensive and quickly prepared, but it also has been shown to be an excellent sorbent of pollutants and supports the bacteria growth [47, 51, 54]. It was noted that the direct utilization of naturally suppressive population of microorganisms or manipulation of the environment in SSF might accelerate effectiveness and provide a renewable control method for the future sustainability [10].

1.5 Anaerobic microbial iron reduction

Iron is a ubiquitous element in the natural environment. It exists in two oxidation states, ferrous (Fe^{+2}) and ferric (Fe^{+3}) iron. The oxidation of ferrous iron leads to precipitation of ferric-(oxides) which have poor solubility at neutral pH. This insoluble form is not available for most organisms in the environment (10^{-18} M at pH=7.0) [57]. It usually settles down to the sub-surfaces, and iron-reducing bacteria reduce the accumulation under the anaerobic condition. Ferrous ion released from MIR process has high solubility and diffuses to the oxic zone where the oxidation takes place. In the sub-oxic zone, few bacteria are also capable of oxidizing ferrous ion by coupling with nitrate reduction or by transferring the electron to photosynthetically active membrane components [58-60]. The biogeochemical cycling of iron in natural environments participates in many important biological processes in oxygen-limited zones such as organic mineralization, nitrogen fixation and respiration, photosynthesis.

1.5.1 Anaerobic microbial iron reduction and applications

Iron reducing bacteria (IRB) are referred to microorganisms that are capable of Fe^{3+} reduction in coupling with oxidation of organic matters. They have been widely found in the sedimentary and subsurface environments. The first isolate known as an IRB was obtained in a Canadian oil pipeline in 1979 and was first studied by Obuekwe in his Ph.D dissertation (1980) under name of *Pseudomonas* sp200 [61]. This organism was then extensively studied by Arnold et al., [62, 63] and was later designated as *Shewanella putrefaciens* [64]. After discovering the phenomenon of iron reduction in river bottoms [65, 66], Lovley and Phillip (1988) were able to isolate a gram negative bacteria from freshwater sediment of Potomac River, Maryland, and designated as GS-15, now known as *Geobacter metallireducens* [67]. At the same time, Mayers and Nelson (1988) isolated a facultative anaerobe, designated as *Alteromonas putrefaciens* MR-1, which grew anaerobically under Fe^{3+} as an electron acceptor [68]. There was a similarity between both *Alteromonas putrefaciens* and *Shewanella putrefaciens*. Since then, several comprehensive studies have been conducted in many regions to identify bacterial strains of microorganisms, to understand more reducing kinetics, and to explore more IRB in various environments. A facultative anaerobic gram-negative was isolated in an estuary in New Hampshire, and was designated as BrY strain, which is capable of using H_2 or lactate as the electron donor and Fe^{3+} as the electron acceptor and did not reside in previous described genus [69]. Other iron reducing bacteria were collected from either freshwater, marine, as well as contaminated sediments, yet they mostly belonged to different genera in the phylum *Proteobacteria* [70, 71] except *Geothrix fermentans* [72]. A

more detail polygenetic diversity of IRB that were found in varieties of sedimentary and subsurface environments is presented in Table A.2.

Anaerobically microbial iron reduction (MIR) is a process in which ferric (Fe^{3+}) is reduced to ferrous (Fe^{2+}) coupled with degradation of organic matters mediated by microorganisms. Several observations have shown that different forms organic compounds could be oxidized by coupling with MIR under anaerobic conditions [73-76]. The process has significant effects on iron geochemistry and organic mineralization in soils and sediments where iron is believed to be the most dominant electron acceptor [66, 77]. Although the MIR has been extensively studied in natural environments and labs, this novel process has not yet been investigated in filtration systems for wastewater treatment. Since SSF possesses similar micro-environmental conditions as hyporheic sediments where iron reducing bacteria can be found [78], the MIR can be manipulated to aid in reclaimed water purification with iron coated sand filters.

The MIR commonly occurs in the subsurface, marine and freshwater sediments. At neutral pH, the iron mineral has low solubility. Electron transfer in the MIR requires direct contact growth of IRB onto the iron mineral surfaces [79, 80]. A previous study reported that IRB excreted proteins to mediate the adhesion on hydrous ferric oxide surfaces [67, 81]. In culture, IRB prefers to stay in an aggregated stage rather than a single cell [67]. This characteristic helps IRBs uptake ferric iron particulates in a liquid phase for growth [82]. As mentioned above, diverse species of IRB detected in a variety of sedimentary environments, indicated that sediments are a favorable habitat for these novel organisms. Reducing conditions of iron-rich sediment creates a harsh environment for other bacteria. Researchers reported that MIR inhibited the sulfate reduction,

nitrification, and methanogenic processes [83-85], indicating that activities of iron reducers affected the respiration of other bacteria in anaerobic habitats. Thus, further understanding of IRB activities in treatment systems would aid as a better means of water reclamation.

The interest in MIR has increased greatly over the past several decades. As extensively reviewed by Lovley and Nealson (1993) and Saffarini (1994), microbial iron reduction process shows potential applications in remediation of aquatic and terrestrial environments polluted by heavy metals as well as organic carbons [77, 86]. Previous studies showed that IRB are able to detoxify heavy metals toxic to less or non-toxic forms or to stabilize the mobile form by precipitation in subsurface environments [87-89]. IRB are also capable of using varieties of organic carbon compounds for respiration energy. Degradation of several organic compounds have been observed in contaminated sedimentary and subsurface environments [73, 75, 76, 90, 91]. Figure 1 shows the oxidation pathways of several organic substrates in coupling with Fe^{3+} reduction to form carbon dioxide (CO_2) and ferrous as the end products [71]. In addition to environmental remediation, recent findings have shown that IRB can be used as a bioelectrical wire to harness energy from waste [92, 93].

Although MIR process appears to have potential *in situ* applications in cleaning contaminated sites, it also raises concerns for the both the environment and industry. MIR in an aquifer will cause more problems rather than helping because of the release of soluble ferrous ions into groundwater supplies. When groundwater containing ferrous ion is pumped and comes into contact with oxygen, the ferrous ion is oxidized into ferric oxides that frequently causes clogs in well systems [71]. Additionally, MIR in submerged

soils may release the ferric oxide-bound arsenate on into solutions [94]. Adverse effects of MIR are also observed in food processing where IRB cause corrosion and food spoilage [95].

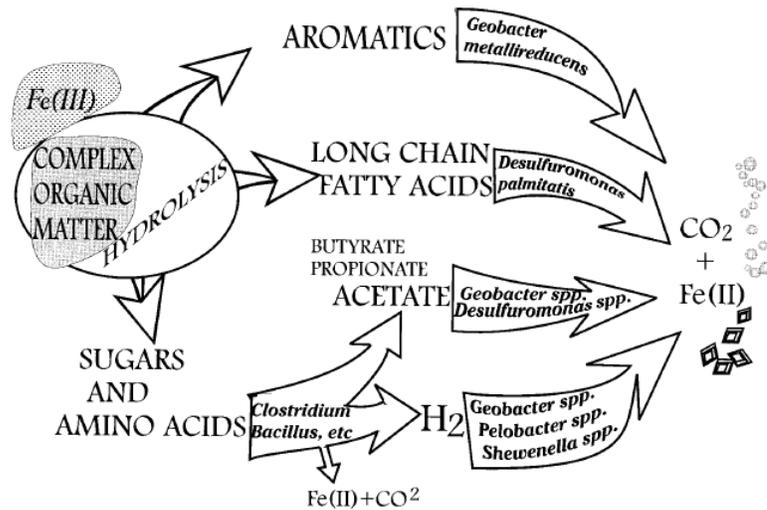


Figure 1.1. Varieties of organic substrates severing electron donor in MIR process in sediments.

1.5.2 Iron and inactivation of bacteria.

Iron is not only an essential trace nutrient for the variety of metabolic pathways, but it is also toxic to living organisms. It is a constituent of many redox-active proteins such as iron-sulfur clusters or all heme groups. The iron-containing proteins play diverse roles in bacterial cells. In starvation conditions, the presence of iron significantly stimulates the proliferation of indicator bacteria [96]. There are different strategies such as excretion of ferric-binding chelator (siderophore), uptake iron sources from the host, or reduction of ferric to form ferrous ion and transport into the cells used to acquire iron [97]. Table 3 shows bacterial species and their strategies of iron acquisition. Although ferrous iron is soluble and available to all organisms, little information is known about

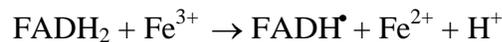
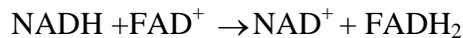
the transport system of ferrous iron in bacteria [97]. Previous research reported that *E. coli* can acquire iron by using *feo* or *fit* systems depending on growing conditions [98, 99]. Under the anaerobic condition, *E. coli* is obtained the ferrous ion via the *feo* system rather than using ferric chelating strategy [98]. The binding site of *feo* system that acts with the ferrous iron is then imported by *feoB* protein localized in the cytoplasm. It is believed that the *feo* system uses ATP as an energy source for transporting the ferrous ion [98]. For aerobic growth, *E. coli* relies on the *fit* system, a putative transporter of ferric or ferrous iron, to acquire iron [99]. The *fit* transport systems de-repressed in limited iron nutrient but repressed in a high concentration of the ferrous ion. Researchers reported that the *feo* system had shown to transport ferric iron while the *fit* system was incapable of transporting iron-containing proteins and siderophore in *E. coli* [98, 99]. Another strategy that aerobic growing bacteria use to obtain iron is to produce the high-affinity iron chelators [97]. The excreted microbial products chelate iron to form iron-siderophore complexes that are imported into bacteria by binding to an outer membrane receptor and subsequently transported into the cellular compartment by several inner membrane-associated proteins. Similar to the *fit* system, bacteria use this production to acquire iron in a substrate-limited condition.

Table 1.3. Strategies of bacteria for iron acquisition*

Strategies	Examples
Use siderophore to chelate ferric iron	Many genera of bacteria
Reduce Fe ³⁺ and transport Fe ²⁺	<i>Legionella spp.</i> , <i>Streptococcus spp.</i>
Directly uptake iron sources from the host (heme, transferrin, lactoferrin)	<i>Neisseria spp.</i> , <i>Haemophilus influenza</i> , <i>Helicobacter pylori</i> , <i>Vibro spp.</i> , <i>Yersinia spp.</i>

*Adapted from Reference [97]

When the imported iron is transferred into the cytoplasm, it incorporates with proteins to become iron-containing proteins in bacterial cells. For example, the intracellular iron is stored in ferritin, bacterioferritin, or DNA-binding proteins from starved cells (Dps) [100-102]. The imported iron may also bind to the surfaces of biomolecules. This iron-binding form is considered to be intracellular free iron. It was reported that *E. coli* mutant (knockout iron regulon Fur) cells contain 300-500 μ L free iron [103]. Another study also showed that the concentration of the intracellular free iron in *Streptococcus* was over hundred micromolar levels under anaerobic conditions [104]. The intracellular free iron mostly exists in a reduced form; however, ferric iron could be reduced by receiving the electron from FADH₂ to form ferrous iron [105].



The Fenton reaction is a well-known process used to oxidize organic compounds as well as inactivate pathogenic bacteria in water and wastewater. The reaction between Fe^{2+} and hydrogen peroxide (H_2O_2) results in the highly oxidative agent, hydroxyl radicals, which cause damage to DNA [106]. When bacteria grow in an iron-rich environment, they may over-import the iron into the cells. As a result, the intracellular free iron is increased accordingly. Elevated intracellular iron have been shown to increase oxidation of DNA in non-respiring cells [105]. In addition to intracellular iron synthesis, bacteria also continuously generate H_2O_2 in cells during the metabolism. Some microorganisms use this metabolic process to outcompete with others in their habitats. Recent observations had shown that *E. coli* cells produced H_2O_2 at the rate micromole levels per second when they grew under the aerobic condition with glucose as an electron donor [107-109]. The H_2O_2 is also generated under the anaerobic respiration. *E. coli* uses fumarate reductase that is synthesized under the anaerobic condition to produce H_2O_2 [108]. Although H_2O_2 is continuously generated in the cells, the bacteria always maintain its intracellular concentration at nanomolar levels. Because H_2O_2 is acutely toxic, bacteria elaborate the defense system by composing different scavenging enzymes to diminish the concentration to sub-lethal level ($<10^{-7}$ M). Researchers reported that the concentration of H_2O_2 inside the cells depended upon the degradation rate by enzymes and leakage through the cell membrane [107]. Alkyl hydroperoxide reductase (Ahp) and catalase are believed to play significant roles to scavenge intracellular H_2O_2 at low to high concentrations [109, 110]. Bacteria also resist oxidative stress by using superoxide dismutase, or peroxidase to scavenge H_2O_2 .

It is possible that Fenton and Fenton like reactions occur outside or within the cells under anaerobic conditions driven by bacteria-synthesized catalysts. The intracellular H_2O_2 may leak through the cell membrane and react with high soluble Fe^{2+} to form the hydroxyl radical. A recent study demonstrated the Fenton reaction was biologically driven under anaerobic-aerobic conditions in alternate intervals [111]. Endogenous H_2O_2 may also react with the intracellular free iron in the cells. It was reported that elevated intracellular H_2O_2 causes damage to critical biomolecules in the bacteria cells [112]. However, it is unknown if Fe^{2+} produced up to millimolar levels during MIR process under anaerobic condition with varieties of electron donors [67, 74] could react with leakage H_2O_2 generated by *E. coli* in aerobic growth [107]; or the intracellular H_2O_2 will react with the intracellular free iron from over imported in ferrous-rich environment. This presumption may lead to inactivation of *E. coli* or pathogenic microorganisms when they enter iron reducing environment. Although recent studies showed that *E. coli* and MS2 were inactivated under anaerobic condition when ferrous ion was amended [113, 114], the gap of knowledge pertaining to activities of IRB and Fenton reaction in a biological system to inactivate pathogenic bacteria will hamper the potential application of the novel biogeochemical process.

1.6 Objectives and structure of dissertation

To reduce the environmental problems and potential health risks, most of wastewater treatment and related research for water reclamation have been focused on technical issues to improve the quality of water effluent. However, the advanced technology does not seem to provide a long-term solution for water reclamation due to energy intensive. At the same time, low-cost treatment methods show promise in

removing contaminants from wastewater for some levels, but more investigation of these treatment means are still needed to improve efficacy. Therefore, a new approach focusing on biological aspects of iron-rich soil filtration was proposed for the long-term and sustainable water reclamation. The overall objective of this dissertation was to explore the microbial activities as a means to remove the contaminants from agricultural and domestic wastewater. Hawaiian soil and ferric oxyhydroxide (FeOOH) coated sand were used as the iron-rich soil based filtration media. Different factors such as bacterivory by protozoa, biofilm adsorption, degradation, and oxidative stress were assumed to play significant roles in removing contaminants. Understanding the contribution of these factors in bacterial removal helps to improve the effectiveness and feasibility of low-cost soil based technology for water reclamation.

This dissertation is divided into six chapters.

Chapter 1 presents the general overview of water reclamation, issues, and low-cost treatment methods, microbial iron reduction, and its potential applications.

Chapter 2, 3, 4, and 5 present four specific research objectives which include introduction, materials and methods, and results and discussion.

Chapter 6 provides a summary of the findings and recommendations for future studies.

CHAPTER 2. IMPROVING SOIL FILTER COLUMN FOR REMOVING BACTERIA FROM SWINE WASTEWATER

Abstract

Wherever there is economic development, there is often a measurable decline in environmental quality. The increasing of swine production in Pacific Islands inevitably leads to environmental concerns of discharge of wastewater that derived from washing and manure. The slurry is accumulated in lagoons whereas supernatant wastewater containing high levels of pathogens and nutrients becomes non-point source water pollutants that deteriorate the quality of coastal water and other water catchments. Soil filtration is promising of a cost-effective technology in removing pollutants from swine wastewater, but the high variation of bacteria removal is often observed during the filtration process. This study investigates an improved soil filter mediated by protozoa activities to remove *Escherichia coli* (*E. coli*) in synthetic swine wastewater. We hypothesized that increasing positive charge of filter media will increase the bacterial attachment and thus stimulates the protozoa grazing of the absorbed bacteria in the filter. The experiments were conducted using mini plastic columns packed with engineered Leilehua soil from Oahu Island, Hawai'i. Our results showed that 95.5% to 96.5% of the *E. coli* were from the influent by physicochemical soil adsorption. The average removal efficiencies were increased to 98.1% in a single stage and 99.99% in two sequential columns under bacterivorous conditions. The experimental data suggest the protozoa bacterivores in an improved soil media create a stabilized bioactive filter to remove *E.*

coli from the influent. The information of this study may be useful for designing a scale-up system using local soil for practical applications of swine wastewater treatment.

2.1 Introduction

Developing and implementing economic and sustainable swine waste management and treatment strategies in the Pacific Islands are critical issues that will determine the fate of the swine industry. Swine manure production generally contains high levels of organic matters, nutrients, and pathogens, and thus, it can be considered as a nutrient source for agricultural practices as well as non-point source pollution from runoff and seepage. The traditional disposal method of swine manure primarily removes solid content via anaerobic processes and is subsequently followed by sedimentation in lagoons. However, supernatant that contains high levels of contaminants is often discharged into streams and violates the aquatic systems or is reused to irrigate crops. The greatest concern of swine wastewater is the pathogenic bacteria with a high level of antibiotic resistance [115]. A review by Guan and Holley (2003) concluded that pathogens derived from animal manure can survive in different environmental conditions and can cause a variety of illnesses in humans, animals, and livestock [116]. Although the quality standard of effluent has been established, farmers have difficulties in attaining this standard due to economic constraints. With increasing environmental concern for health risks relating to the Clean Water Act, investigations in inexpensive pollution prevention technology to remove pathogenic bacteria from animal farming effluent requires our immediate attention. Cost-effective pollution prevention technology will be beneficial to both agricultural practices and the health and safety of the environment and those who live in it.

Cost-effective technologies for agricultural wastewater treatment processes have attempted to explore in practice and academia to both improve discharge quality to the environment and to merit community confidence. The objective of wastewater treatment is to remove contaminants using technologies that have been proven to be successful. Several treatment methods are available to remove contaminants from water and wastewater. The appropriate choice of technologies wholly depends on pollutants, required standards, infrastructure, and cost. In any case, sustainability of water use should be our major target to protect the water sources because it will reduce the water quality issues and thus enhance the aquatic ecosystems. Low-cost treatment efforts that exploit the natural processes and are often used in rural areas of developing countries reduce some levels of contaminants. Although water quality regulations are not strictly enforced, these efforts are still not accepted as an appropriate process to remove pollutants from agricultural wastewater due to elevated health risk derived from pathogens. The wetland system is a traditional technology that is a low-cost investment and requires little maintenance. Constructed wetlands have been used successfully to remove organic matters and nutrients from domestic wastewater in developing countries [117, 118]. Modifications or combinations of wetland systems that were used to treat domestic and discharge of dairy wastewater had a high removal rate of organic carbon and nutrients [119, 120]. Inexpensive filtration systems that use cheap and locally available materials from nature and waste byproducts also provide a high removal percentage of phosphate via the adsorption process [23, 121]. Another cost-effective technology is to reduce energy usage during extensive aeration in wastewater treatment by improving aeration efficiency for activities of active biofilm in aerobic microbiological processes [122].

Soil filtration has been cited as a potential process to remediate wastewater. Numerous studies have documented that soil filtration systems reduced levels of nitrogen, phosphorus, organic carbon, and microorganisms from wastewater [21-23, 123-125]. Different designs and operational conditions of soil filters and characteristics of influents resulted in the different removal rate of contaminants. It was reported that soil columns effectively removed viruses from the treated wastewater with a filter depth at least 80 cm [22, 125]. Increasing the flow rate led to a reduction of virus removal efficiency in the soil filter columns [125]. The Multi-Soil-Layer (MSL) system packed with Leilehua soil potentially removes a high percentage of phosphate and organic nitrogen from dairy effluent to meet the requirement of Hawaii Department of Health [23]. However, this technology is still not accepted as a means to treat wastewater in the United States because of its critical inability to produce water that meets either State or National Standards. A particular concern of this treatment method is the high degree of variability in the removal of bacteria.

The fate and transport of microorganisms through porous media have been extensively studied over the past few decades due to its significant relevance to drinking water supply protection, in situ remediation and wastewater treatment. Several studies demonstrated the transport and adsorption of microorganisms in columns packed with cheap materials such as sand [30, 123, 126-133], glass beads [134], soil [22, 123, 125, 130, 135], or synthetic media [136]. Physical and chemical properties of solid and liquid phase and bacteria including solution chemistry, fluid velocity, grain collector size, surface roughness, surface charge all affect the bacterial transport [130, 133, 137]. Biological factors such as cell type, growth stage, concentration, extracellular polymeric

substances, and biofilm were also considered as influencing factors [123, 126, 127, 131, 132, 136, 138]. Bacterial retention in porous media was caused by adsorption mechanism due to the physical- chemical interaction of bacteria surface properties and solid phase [126, 128, 136]. Accumulation of microorganisms was found in the gas-water interface of unsaturated media [136, 139]. Biofilm formation due to bacteria-solid and bacteria-bacteria interaction also influenced the transport of bacteria through porous media [129].

Bacterial adsorption in soil media may depend on physical and chemical properties of the soil. A previous study addressed that different soil types did not significantly affect retention of bacteria, but the acidic soil was documented as a better medium for bacterial adsorption than alkaline soil [135]. Increasing the positive charge surface of filter media enhanced bacteria adsorption [40, 140]. Continuous transport of bacteria through soil columns results in increasing bacterial concentration in the associated porous media. The more the bacteria attach to the solid surfaces, the faster bio-clogging occurs in the filtration media. However, the depositing of motile bacteria provides more favorable means of collection than that of the non-motile bacteria [130]. Surface collectors were reduced, and adsorption sites were blocked at equilibrium condition for non-motile bacteria. Consequently, retained bacteria could be washed out of adsorption sites to reduce blocking sites [135], and, therefore, it may cause inefficiency in removing bacteria in column system during operation.

Biological interactions play a significant role in the regulation of bacterial populations in environmental microbial ecology. Modified bacterial composition in soil and levels of microbial activities lead to increasing competition and antagonism among microbes by adding more substrates [141, 142]. Protozoa are known as predators in soil

and water environments that can regulate bacterial populations [143-148] in wastewater treatment systems [149-151]. The addition of *E. coli* to soil caused an increase in the population of indigenous soil protozoa [143]. The presence of protozoa was attributable to the *E. coli* reduction in estuarine water [152]. In soil environments, *Colpoda stinii* was identified in the regulation of *Pseudomonas fluorescens* populations [153]. However, a previous study also reported that the response of protozoa to the reduction of other soil bacteria was not significant [143]. In bioreactor systems, the reduction of protozoa populations resulted in an abundance of assimilated organic bacteria [150]. According to Gonzale et al.(1990), protozoa grazing caused the elimination of *Enterococcus faecalis* [144]. Decamp et al. (1999) reported that the moderate grazing rate of protozoa ranged from 9.5 to 49 bacteria/protozoa/hour for the planted and unplanted bed wetland [149]. Protozoa was also found to have different ingestion and digestion rates for different types of bacteria in different environmental conditions [144]. As a point, in fact, protozoa have a significant impact in regulating bacterial populations in natural environments.

The integration of increasing bacterial adsorption by positive charge rich soil media and protozoa bacterivory in a filtration system may provide a cost-effective and sustainable approach in removing pathogenic microorganism from swine wastewater. Soil particles retain the mobile bacteria on to surfaces and native protozoa grazers enhance bacterial removal efficiency of the filter. The Hawaiian Islands are volcanic in origin, and thus the soil content is rich in iron oxides that promote the electrostatic interactions with a negative charge of bacterial cells. Nevertheless, little information is available about the removal of bacteria while passing through this particular natural media. Our objectives were to examine the bacterial removal in Leilehua soil filters and

the roles of indigenous soil protozoa as an active biological factor for improving the efficiency. An engineered strain of gram-negative bacteria, *E. coli* ATCC29522, was selected as the model bacteria. Series filtration experiments were conducted in a laboratory scale filtration system to study the effects of bacterial adsorption and predation in soil filters.

2.2 Materials and methods

2.2.1 Filtration media preparation and experiment setup.

The soil column experiments were performed in polyvinyl chloride (PVC) pipes (inner diameter: 10.1 cm; length: 38 cm). Leilehua soil was sieved to select particles with sizes ranging from 7.0-8.0 mm. Perlite sieved to the same size as soil was coated with a thick layer of ferric oxide (FeO) using the method adapted from the Pi strip methodology [154]. The mixture comprising one part FeO coated perlite, and three parts soil were then dry-packed into the columns by batch pouring to achieve a consistent final media depth 38 cm. Before starting experiments, all soil columns were initially water saturated by feeding 0.01M CaCl₂ salt solution for overnight with flow rate 8.0 L/day. Artificial swine wastewater (N: 750 mg/L, P: 75 mg/L, K: 750 mg/L, Ca: 100 mg/L, Mg: 25 mg/L, Na: 150 mg/L) was prepared based the actual constituents of swine wastewater in Oahu, Hawaii Islands. The artificial effluent was fed into the columns using peristaltic pumps with flow rate 8.0 L/day. The flow rate amounted to a volumetric loading of c.a. 100 mL/cm².day, through an inlet port located at the top of the columns. Effluent gradually drained out by gravitational force at the bottom of soil columns via an outlet tube.

2.2.2 Microorganisms and culture.

E. coli strain ATCC 29522 was used as the model organism in the experiments. Fresh stationary-phase cells were prepared by inoculating fresh overnight single colonies from TSA agar plates into LB broth, growing at 37°C with continuous agitation, and harvesting at stationary phase ($OD_{600} > 1.2$). The collected cells were centrifuged at $10,000 \times g$ for 3.0 minutes followed by washing with phosphate buffer water (PBW) for three cycles. The harvested bacterial cells were suspended in PBW to make a stock solution with an approximate concentration of 10^9 - 10^{10} CFU/mL ($OD_{600}=0.6$) to prepare the working solution for experiments. The media containing suspended bacterial cells was kept at 4°C to minimize cell growth or decay during experiments. In our experiments, no marked growth or decay of bacteria in the influent were observed.

2.2.3 Capability of improved soil filter for *E. coli* removal by physicochemical adsorption

The removal of *E. coli* to the soil filtration media by physicochemical adsorption was examined by continuously applying two different levels of bacterial concentration into the soil columns. The high input influent concentration was 10^8 CFU/mL, and the lower level was 10^6 CFU/mL; that correspond to the loading rates of 10^{10} CFU/cm².day and 10^8 CFU/cm².day. The capability of improved soil filter for bacteria removal was determined by comparing the elimination rate for two different loading rates.

2.2.4 Protozoa growth and bacterial removal in soil column

A microcosm study used the same soil was set up to investigate the protozoa growth and bacterivory in response to the supply of *E.coli* cells as prey in the soil. Six mini soil columns were divided into two sets; one set was periodically treated with 200

mg/L of cycloheximide (i.e., cycloheximide-treated or CT) to inhibit protozoa growth and reduce protozoan activities, while the other set was not perturbed by cycloheximide (i.e., natural protozoa growth or NPG). At this level of inhibiting factor, protozoa in soil media did not survive [155]. All filter columns were fed with 2.0 L/day 0.01 M CaCl₂ solution containing approximately 10⁵-10⁶ CFU/mL of *E. coli* cells, which was significantly higher than the native *E. coli* population density found in Leilehua soil (<10 CFU/g). The CaCl₂ solution with no nutrient minimized any unexpected growth of bacteria and protozoa during transport of bacteria through soil columns. Effluent was drained continuously through an outlet at the bottom of soil columns, and the concentration *E. coli* cells were determined every in 8 hours intervals.

2.2.5 Effects of protozoa activity on bacterial removal

Effects of protozoa multiplication on *E. coli* removal were investigated in replicated soil filter columns with two treatments. One treatment was soil columns with pre-enriched protozoa (PEP) population by amending nutrient source (50 mg/L of sucrose) in feeding water solution. The soil columns were continuously fed to stimulate the growth of indigenous soil protozoa. After enrichment, the protozoa population increased up to levels of 10⁴-10⁵ MPN/mL in the effluent solution. Another treatment was soil columns with natural protozoa growth (NPG) in responding to the introduced bacteria. Soil columns of both treatments were continuously fed with artificial swine wastewater with a concentration of *E. coli* at approximately 10⁵-10⁶ CFU/mL. Effluents were collected in one-liter plastic bottles for 2 hours at the outlet every day over the course of the experiment.

The abundance of indigenous soil protozoa and absorbed *E. coli* in soil media were also determined at the end of the experiments. All experimental soil filters were interrupted after 20 days, and soil samples were collected at different depths once the effluent had been drained from each of the columns. Soil profiles were cut into five equal sections along the depth. All samples were immediately processed to quantify the numbers of active protozoa and trapped bacterial cells in the soil media.

2.2.6 Quantification of microorganisms

a. *E. coli* quantification

Water and soil samples were processed immediately after collection. *E. coli* in influent and effluent were enumerated using membrane filtration method. For water samples, a serial dilution from 10^{-1} to 10^{-5} were prepared by transferring 1.0 mL to 9.0 mL of sterile phosphate buffer saline (PBS). Soil samples were prepared the same manner as water samples. Wet soil, samples were weighed out to 5 grams and then suspended in 45 mL of sterile deionized (DI) water (10^{-1}). A subsequent transfer of 1.0mL to 9.0 mL of sterile PBS buffer was made to establish the serial dilutions. Ten milliliters of the aliquot dilutions were then filtered through 0.45 μm sterile GN-6 membranes (Pall Life Science, Port Washington, NY). The membranes were then placed on modified membrane thermotolerant *E. coli* agar (mTEC) which contained necessary nutrients for *E. coli* growth. All culture plates were then incubated in water bath at 35°C for 2 hours and overnight at 44.5°C.

b. Protozoa enumeration.

Protozoa in water samples were quantified by using most probable number (MPN) method [143]. Serial dilutions from 10^{-1} to 10^{-4} were prepared by transferring 1.0

mL of samples to 9.0 mL of Page's amoeba saline (PAS) buffer. For soil samples, the protozoa population was quantified using MPN method described in the previous study [156]. 10 g of wet soil samples were suspended in 90 mL of sterile DI water containing in a 250 mL flask (10^1) and agitated for 3 minutes. A serial dilution was made by subsequently transferring 1.0 mL of suspension into 9.0 mL of PAS buffer to establish a serial dilutions from 10^{-2} to 10^{-5} . The mixtures were then used to prepare five-fold MPN serial dilutions in 96-well plates. *E. coli* was used as food for protozoan growth. 20 μ L of *E. coli* with a concentration of 10^8 - 10^9 ($OD_{600}=0.4$) as an only prey source was added into microtiters containing 100 μ L of aliquot dilutions. The ratio of prey and predator for protozoa recovery was 1:5 [143]. The culture plates were incubated in the dark at 10°C for 1-3 weeks and were periodically examined for the presence or absence of protozoa using an inverted microscope.

2.3 Results

2.3.1 Adsorption of *E. coli* into Leilehua soil

Figure 2.1 shows the removal of *E. coli* by improved soil columns. The effluent *E. coli* concentration from the high bacterial loading rate gradually increased after 6 hours of feeding, suggesting that the bacterial adsorption was being gradually limited overtime. Similarly, the effluent *E. coli* concentration from the low bacterial loading rate also increased overtime. However, the columns have an initial ease of bacterial adsorption at the onset, but its capacity to absorb was exceeded when more bacteria were loaded.

The average removal efficiencies of *E. coli* in improved soil columns for high and low bacterial loadings were 92.8 % and 98.6 %. The soil columns feeding lower bacterial

concentration seemed to have the greater extent of *E. coli* removal than that in the case of the high input. However, there was no statistically significant difference between two loadings. The removal of *E. coli* was found not to be stable during the experiments. The preliminary results indicated that the soil media may have adsorption affinity to bacteria, but there might be the finite capacity to retain bacteria in the soil columns.

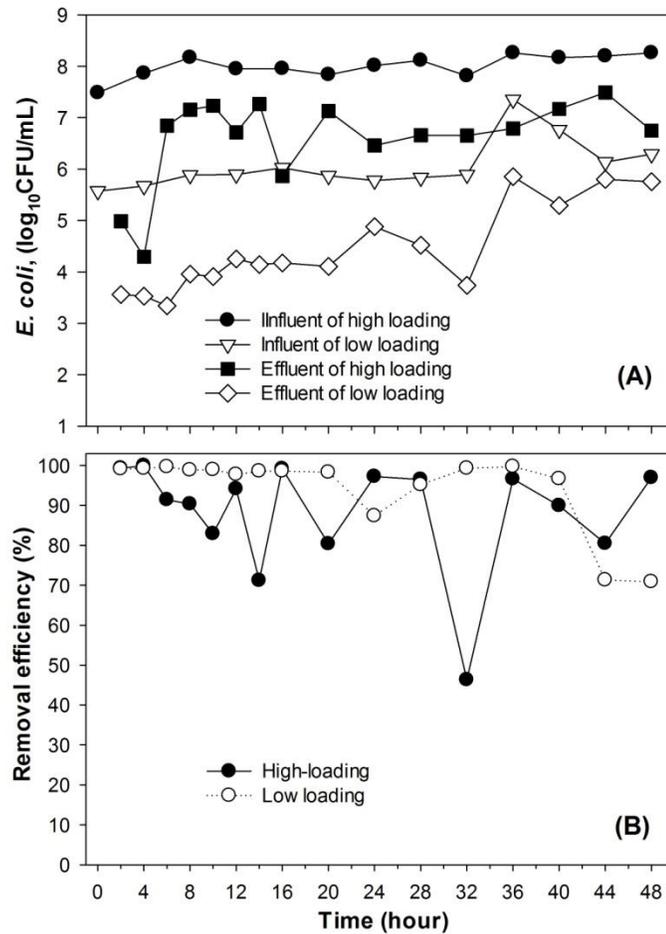


Figure 2.1. Influent and effluent concentrations (A) and removal efficiencies (B) of *E. coli* in Leilehua soil applying at two different loadings of bacterial concentration.

2.3.2 Protozoa response to the addition of *E. coli*

The recovery of indigenous soil protozoa by nutrient sources from absorbed *E. coli* was examined in mini soil columns. Protozoa initiated growth when the soil columns were fed with a CaCl₂ solution containing *E. coli*. Figure 2.2A shows that the indigenous protozoa in Leilehua soil were recovered in the columns as indicated by the detection in the effluents after four days. This result suggested that the native protozoa used the retained *E. coli* as food for growth. Continuously applying *E. coli* into the columns stimulated the proliferation of protozoa. However, low numbers of protozoa were detected in the column effluent water. It was possible abundant protozoa tend to reside within the soil media where there were plenty of trapped *E. coli* serving as the food source. In contrast, there is no detection of protozoa in the effluent water of CT columns, suggesting that physicochemical factors were attributable to the *E. coli* removal.

Protozoan bacterivory in soil media helped maintain higher removal rate of *E. coli* than that without protozoa. Analogous to the preliminary results, efficient *E. coli* removal was achieved in the soil microcosm columns. The introduced bacteria were consistently removed for both treatments. There was 99.99 % feeding bacteria retained in soil filtration media. However, the removal of *E. coli* dramatically declined in the soil columns with the absence of predator after 7 days (Figure 2.2B) when bacteria were continuously loaded into the system. The reduction of removal rate might be due to decreasing adsorption sites while more bacteria were passing through filtration media. In contrast, the NGP columns maintained a stable efficiency. The higher *E. coli* removal rate in natural growth after the first-week operation could be due to a combination of

physicochemical adsorption and predation by protozoa. The captured *E. coli* in soil media served as the food supply for the indigenous soil protozoa.

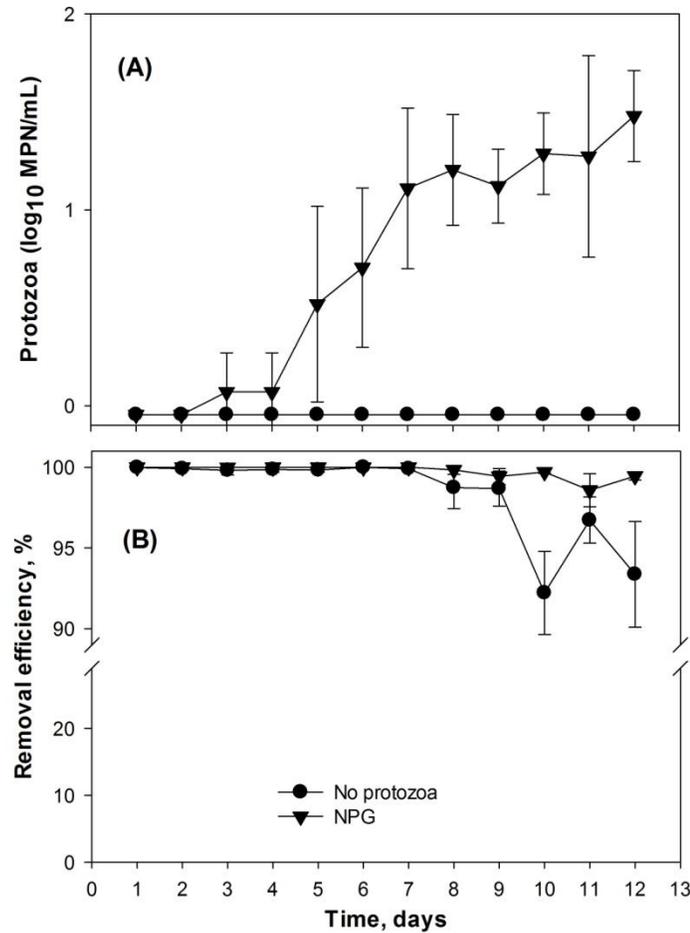


Figure 2.2. Protozoa growth (A) and *E. coli* removal efficiencies (B) in treated and untreated column by cycloheximide.

2.3.3 Effects of the presence of protozoa on removal efficiency of *E. coli* in soil columns

The third experiment was conducted to compare grazing rates between the pre-enrichment protozoa (PEP) and natural protozoa growth (NPG). The results showed that the high percentage removal efficiency of *E. coli* was achieved in both PEP and NPG treatment columns (Figure 2.3A). However, NPG columns most likely had fluctuation at

the initial time of operation. Because active protozoa did not recover yet, adsorption sites of soil media might exceed the limits to mediate the continuously fed bacteria. The soil columns with pre-enriched indigenous protozoa consistently reduced *E. coli* overtime. The average removal efficiencies for the entire course of the experiment were 90.9 % for the PEP treatment and 87.3 % for the NPG treatment. Our results suggested that protozoa ingested the trapped bacteria in soil media and helped the soil columns maintain the more stable *E. coli* removal rate.

The numbers of protozoa in treated wastewater increased dramatically after four days (Figure 2.3B), suggesting that protozoa had naturally recovered when *E. coli* were applied into the in the soil columns. The results of this experiment aligned with the results of the microcosm experiment. Initially, the protozoa numbers detected in the treated wastewater were very small, yet the concentration increased and reached a stationary level. The protozoa growth in this experiment reached a steady state faster than that in the microcosm experiment (observed by the effluent concentration). Prey-predator mechanisms affected the changing dynamic of both bacteria and protozoa populations could be a possible explanation. The result of this experiment consistently demonstrated that indigenous protozoa grazing played a significant role in the removal of *E. coli* from wastewater in the soil column system. Overall, there was no significant difference in the *E. coli* removal efficiency between the pre-enrichment and natural recovery of protozoa. However, protozoan community stimulated by nutrient source absorbed *E. coli* seemed to be an active predator. Protozoa concentration associated with wastewater solution passing through the soil columns.

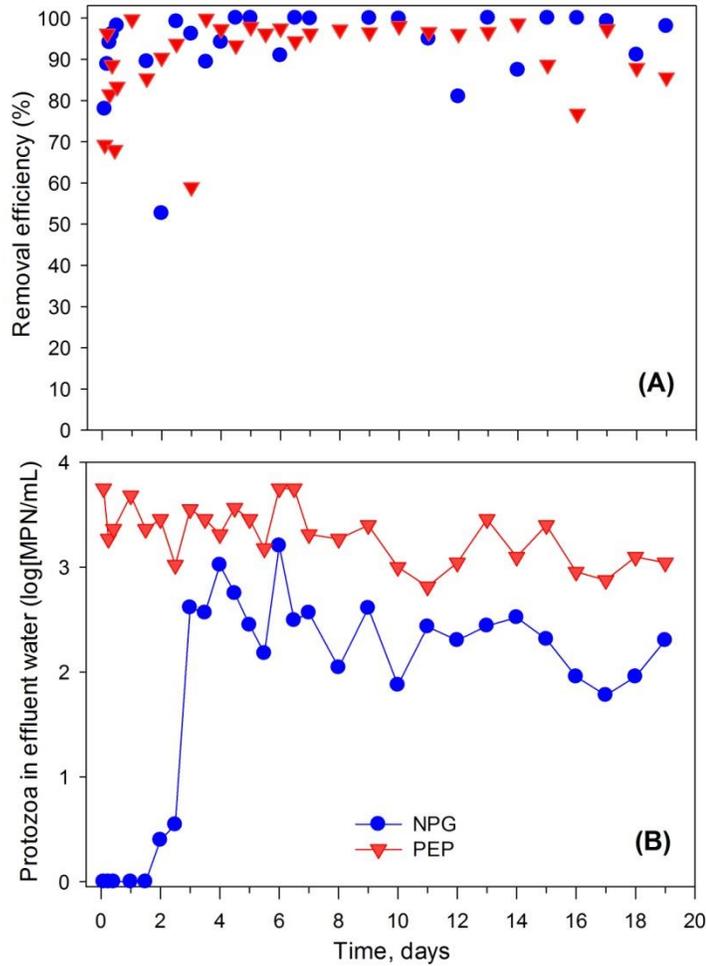


Figure 2.3. *E. coli* removal efficiency (A) and protozoa in effluent water in PEP and NPG filters.

2.3.4 Abundance of protozoa and bacterial adsorption in the soil column

The soil columns were interrupted at the end of the experiment, and two representative soil samples from replicate columns were collected at five different depths to examine the distribution of active protozoa and captured bacteria in filtration media. The numbers of predator and prey were quantified at five different depths of the soil profile. Figure 2.4 presented the residing protozoa and absorbed *E. coli* in the soil media with respect to filter depth. As shown in Figure 2.4A, there were high numbers *E. coli*

retained on the top filtration zone, suggesting that bacteria were most likely captured by the soil particles when the effluent flow was passing through the media. This experimental result again suggested that Leilehua soil attracts and retains bacteria during the filtration process. At lower filtration depth, less *E. coli* were detected in the soil samples. The lower concentration of *E. coli* at the bottom of the columns was the result of both physicochemical removal of *E. coli* and elimination of *E. coli* by predators in upper filtration zones. In comparison, the NPG columns captured more *E. coli* at the top while PEP columns seemed to have less *E. coli* adsorption in the respective location. As a result, more *E. coli* were captured in the upper filtration zone than that in the bottom. The difference between bacterial adsorption to the soil media in the two treatments was probably influenced by physicochemical changes of soil properties in the pre-enrichment process. Therefore, the adsorption affinity to the *E. coli* was reduced.

The bacterivores colonizing along the filtration depths respond to the presence of prey. Figure 2.4B shows the distribution of protozoa population along the soil filtration depths. The protozoa were abundant in the upper portion of filtration media where there was plenty of food. In contrast, fewer numbers of predators were detected at the bottom zones. The reduction of protozoa population might be attributed to the availability of nutrient sources from the *E. coli*. There was a high correlation between the numbers of predators and the distribution of captured *E. coli* along the depth.

2.3.5 Enhancement of bacterial removal in two stage filtration

The MSL swine wastewater treatment has shown to reduce *E. coli* in the influent. However, only a small fraction of the fecal bacteria was removed by our testing columns,

and thus the concentration in the treated water did not meet the standard level requirement of Hawaii Department of Health. Therefore, a system adjustment was made to investigate whether the previous design and operation affected the removal efficiency. Figure 2.5 presents the results of bacterial removal in the two sequential columns. Surprisingly, the removal efficiency of *E. coli* reached 99.992 % when two MSL columns were placed in sequence and input levels of *E. coli* reduced to 10^4 - 10^6 CFU/100mL. The *E. coli* concentration in the treated water was less than 10 CFU/mL, suggesting that the two sequential MSL columns reduced *E. coli* to ideal target levels.

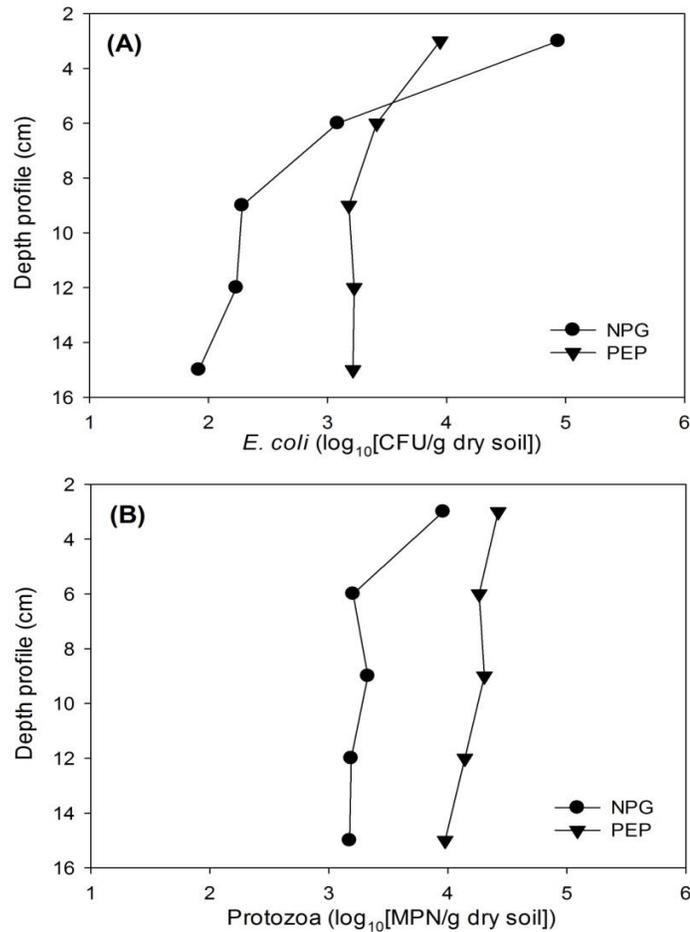


Figure 2.4. Absorbed *E. coli* in the soil media (A) and Protozoa abundance (B) in PEP and NPG soil columns with respect to filter depth.

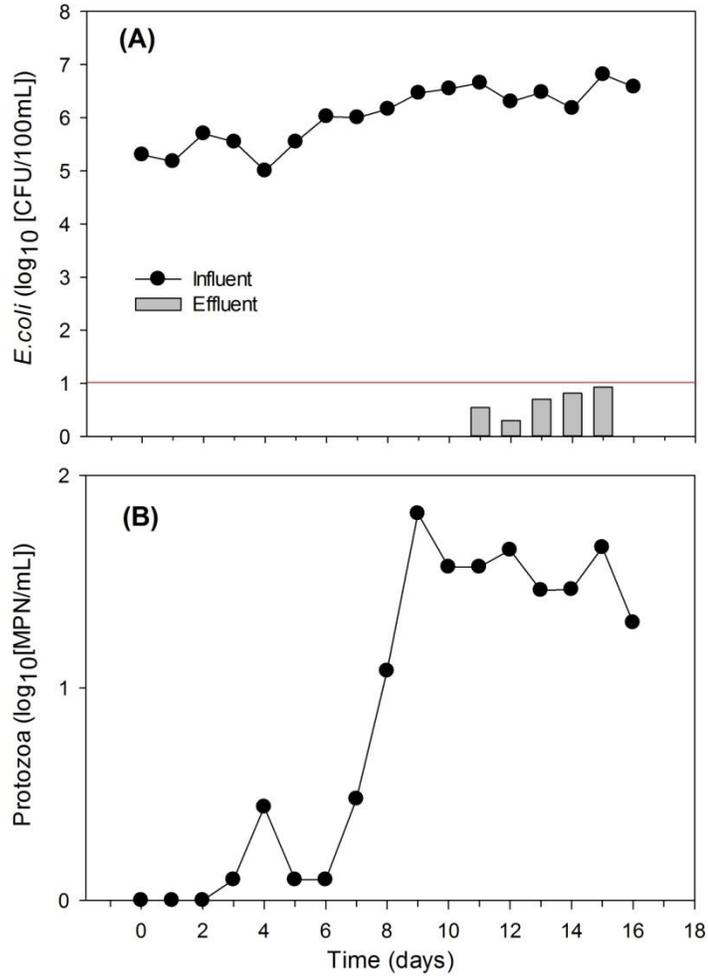


Figure 2.5. *E. coli* in influent and effluent (A) and protozoa detection in treated wastewater (B) in the two sequential soil columns.

2.4 Discussion

2.4.1 Adsorption capacity of Leilehua to *E. coli*

Leilehua soil filters effectively removed phosphates and inorganic nitrogens in dairy effluent wastewater [23]. The high content of ferric oxide in the soil may be attributable to increasing bacterial attachment, and thus stimulates protozoa grazing of attached bacteria because this soil type had showed to strongly absorb the negative charge ions [23, 121, 124]. Bacteria are like the organic particles and carry a negative

charge. Numerous studies documented that the metallic oxides that carry a positive charge actively attract and immobilize the bacteria in porous media [38, 39, 54, 140]. The electrostatic interaction between the two increases the retention of microorganisms in the iron-oxide containing filtration media [38]. Increasing the fraction of iron coated sand in the filtration system led to the enhancing attachment of bacteria to the positively charged surfaces [157]. Our results showed that Leilehua soil removed *E. coli* to some extent, but a high variation of removal efficiency was observed (Figure 2.1). These primarily experimental results were in alignment with a previous study in which fecal coliform was reduced in the multi-soil-layer (MSL) system using Leilehua soil [23]. Although the iron-containing filtration media was shown to improve bacterial retention, it was found that there was a finite adsorption of bacteria to the iron-oxyhydroxide-coated sand [140]. When bacteria entirely cover the soil particles, the positive charge on the surfaces may balance the negative charge of the bacteria. This assumption may suggest that cell-cell interaction or bacterial aggregation may play a major role in capturing the bacteria when soil adsorption sites are not available. Deposits of the motile bacteria increased the overall retention of bacteria while the non-motile bacteria tended to block the adsorption sites [130]. However, excessive colonization of the bacteria on the surface in porous led the bio-clogging [134]. When the columns continuously fed swine wastewater, only a small fraction of bacterial adsorption could be achieved. Therefore, the Leilehua soil probably has a finite capacity to retain bacteria.

2.4.2 Indigenous soil protozoa and bacterial regulation by protozoa

The addition of *E. coli* to the soil resulted in an increase of the native soil protozoa population [143]. Furthermore, the discharge of wastewater containing fecal

bacteria into the stream also lead to responding of free-living protozoa that then determine the bacterial levels in the aquatic systems [158, 159]. Our experimental data showed that indigenous protozoa grew in Leilehua soil filters after three days (Figure 2.2A and 2.3B) when artificial swine wastewater was continuously applying to the columns. The numbers of protozoa detected in the treated effluent at a steady state were 10^2 - 10^3 MPN/mL. However, the protozoa population that colonized in the soil filtration media was higher than that in the effluent water. A previous study reported that the protozoa numbers increased approximately 150 fold after three days incubation in soil microcosms containing *E. coli* [143]. It was very interesting that there were high numbers of protozoa residing at the top media portion of the columns, suggesting absorbed *E. coli* might be attributable to protozoa multiplication and colonization. The protozoa abundance in the soil media highly correlated with the absorbed *E. coli* along the filtration depth (Figure 2.6A). This predator-prey relationship demonstrates that the presence of bacterivores may impact on the flux of bacteria in swine effluent wastewater applying to a pool of protozoan community. However, this study did not provide any direct evidence to support this assumption. The sole clue for the role of protozoa in regulating the bacteria in soil columns was the removal efficiency. For the pre-enrichment soil columns, there was an unlikely correlation between pre-enrich protozoa population and concentration of absorbed *E. coli*. A possible explanation is due to the existing alternative nutrient for protozoa remaining from the enrichment process. Together, the results suggest that the bacterial feeding protista were actively responding to the absorbed prey while pre-enrichment community by the organic substrate was unlikely active to graze the bacteria

for energy. As a consequence, the established protozoa community grazes on the retained bacteria and eliminates them from wastewater, leading to improvement of treated water.

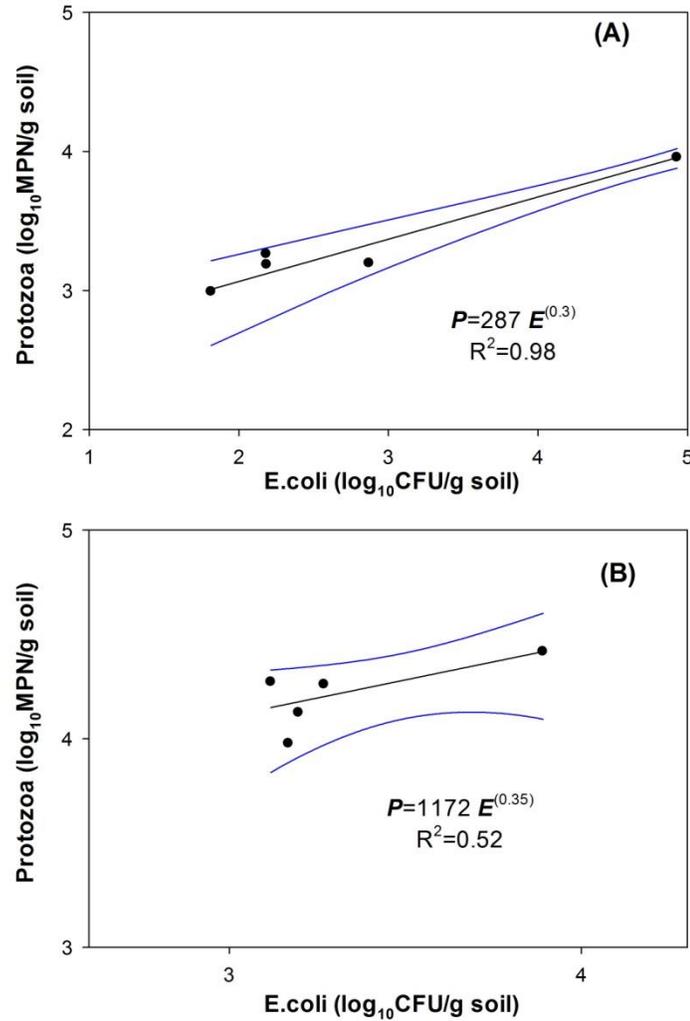


Figure 2.6. The correlation between protozoa abundance and attached *E. coli* along the filter depth of (A) NPG columns and (B) PEP columns.

2.4.3 Effects of protozoa on *E. coli* removal efficiencies in the soil columns

Grazing mechanism results in the microbial population shift of predators and preys in soil and aquatic system [145, 147]. The protozoan grazing also affects the interactions between bacteria and other microorganisms in the soil [143, 147]. This study

showed that the presence of bacterivorous in the soil columns stimulate the more a stable removal efficiency of fecal bacteria than the absence of protozoa predation (Figure 2.2B). The clearance rate of protozoa to fecal bacteria obtained from the fitting classical model was 20 μL per predator per hour [158]. A report cited by Schlimme et al. (1997) revealed that the protozoa consumption rates were 9 to 266 bacteria per hour for a flagellate and 200 to 5000 per hour for a ciliate [160]. However, there is little information available to support the percentage of bacteria removal by protists in a filtration system. A previous study showed that the ciliates ingested the attached bacteria at the rate of $1,382 \pm 1,029$ cells predator⁻¹ hr⁻¹, but the grazing rate reduced approximately one quarter in an infiltration system [161]. The mass balance calculation with the assumption of unremarkable cell death during the experiments showed that the protozoa grazing rates in the replicate Leilehua soil filters were 157 cells protozoa⁻¹ hr⁻¹ of the NPG columns and 35 cells protozoa⁻¹ hr⁻¹ for the PEP columns (Table 2.1). These grazing rates are much lower than the values reported by Eisenmann et al (1998)[161] but higher than that in wetland systems [149]. There was greater grazing in the NPG than that in the PEP, inferring that there more active predators in the NPG filters than that in PEP filters. However, the removal efficiencies between these two treatments were not significantly different. Although the PEP might be less active, the higher population could eliminate *E. coli* in amounts similar to the NPG. Numerous studies have documented that predation mechanisms play a significant role in reducing the bacteria in water and wastewater filtration systems [30, 40, 162, 163]. However, there are different grazing rates obtained from the laboratory scale and field experiments.

Table 2.1. Mass balance of the *E. coli* and protozoa grazing rate in the MSL mini-columns.

Treatment	Total mass <i>E. coli</i> in water, CFU		Total mass absorbed <i>E. coli</i> in soil, CFU			Total protozoa in soil column, MPN	Grazing rate (<i>E. coli</i> / protozoa.hr)
	Influent	Effluent	Retained cells	Live cells	Death cells		
NPG	8.9 x10 ⁹	1.9 x10 ⁹	6.9 x10 ⁹	5.9 x10 ⁷	6.8 x10 ⁹	1.1 x10 ⁷	157
	±2.2x10 ⁹	±1.4x10 ⁹	±7.8x10 ⁸	±1.1x10 ⁶	±7.8x10 ⁸	±1.2x10 ⁶	
PEP	8.9 x10 ⁹	4.7 x 10 ⁸	8.4 x10 ⁹	9.7 x10 ⁶	8.4 x10 ⁹	6.0 x10 ⁷	35
	±7.2x10 ⁸	±7.2x10 ⁷	±7.9x10 ⁸	±6.1x10 ⁶	±8.0x10 ⁸	±9.7x10 ⁶	

2.4.4 Sequential design filters for better removal

Although the MSL mini column with protozoa growth showed to reduce the bacteria, the effluent concentration of testing bacteria was still higher than the standard level of recycled water quality. It was reported that soil filtration often removes bacteria and viruses 2-3 log unit levels [21, 164, 165]. Ion exchange due to building up the alkalinity of the bulk solution and ion exchange of a solution such as Na, K, Ca, Mg, may reduce the bacterial adsorption the soil surfaces. Additionally, naturally occurring heterogeneities in the physical, chemical and microbial properties of the soil mixture and initial non-heterogeneities dry packing process may have also contributed to the variation of bacterial removal efficiency to some extent. Despite the complex sorption mechanism that possibly occurred when a multi-constituent aqueous solution like swine effluent was applied to the soil columns, the absorbed *E. coli* has a non-linear relationship with respect to the filter depth (Figure 2.4A). This is a valid concept in the case soil dispersion.

Previous studies reported that the concentration of fecal bacteria and pathogens in swine effluent were at least 10^5 CFU/100mL [166, 167]. In this study, the bacterial concentration ranged from 10^6 - 10^7 CFU/100mL in the influent. The *E. coli* concentration dramatically increased in the effluent in the single column system. A previous study showed the metallic oxides coated sand filter incorporated with bacterivory predation was efficient in removing pathogenic bacteria [40]. Leilehua soil with iron oxide content was proposed to have adsorption affinity to bacteria. However, continuous loading of microbial contaminants might exceed the finite capacity of the filter columns. In addition, short hydraulic retention time (HRT=4 hours) may also reduce protozoa grazing rate because of shortening contact time. These two factors might reduce the column efficacy

of bacterial removal. By placing two columns in sequence, this limitation was overcome. Thus, a high level of removal rate was obtained in the two sequential MSL mini-columns.

2.5 Conclusions

The MSL mini-columns packed with iron oxide rich soil has shown as a potential treatment means to remove bacteria in artificial swine wastewater. At least 95% of the *E. coli* absorbed to the soil media and was then removed by indigenous soil protozoa. The sequential column design produces a great water quality that meets the R1 standard level of reuse water in Hawaii. The protozoa that naturally grew by feeding bacteria was more active than the pre-enrichment protozoa. This study provides evidence that local soil media is potentially applicable to filter systems to treat the agricultural wastewater. An optimized soil based filter design for the swine water treatment has been proposed for swine wastewater remediation. However, actual swine wastewater that contains high organic solute and colloids should be tested in the same manner because the experiment conducted in this study was using free organic artificial swine wastewater, and it may not be appropriate in the practice.

CHAPTER 3. INACTIVATION OF *ESCHERICHIA COLI* ENHANCED BY ANAEROBIC MICROBIAL IRON REDUCTION

Abstract

Microbial iron reduction (MIR) is an important and ubiquitous natural process in the biogeochemical cycling of iron and carbon in anaerobic sedimentary and subsurface environments. The objectives of this study were (1) to determine if the MIR process can enhance the inactivation of *Escherichia coli* cells under anaerobic conditions and (2) to identify potential inactivation mechanisms. Laboratory microcosm experiments showed that the presence of MIR activity significantly enhanced *E. coli* inactivation, and the inactivation rate under the MIR condition was significantly larger than those under other anaerobic redox conditions. Under the anoxic condition, higher Fe^{2+} concentrations exhibited a linear function to larger *E. coli* inactivation rates, indicating that the production of Fe^{2+} by MIR played an important role in *E. coli* inactivation. When *E. coli* cells were amended as the sole electron source to the MIR process, increased Fe^{2+} production was observed, which corresponded to decreasing TOC concentration. Together, the results suggest that MIR enhanced *E. coli* inactivation through the production of Fe^{2+} as a metabolic waste, and the inactivation benefited the MIR process as the inactivated cells were used as an electron source, which represents a potential new mechanism for bacterial inter-species competition. This knowledge could further improve our understanding of the fate of fecal bacteria in natural environments where the MIR process is prevalent, and may also be explored for the enhanced removal of bacterial pathogens in engineering processes.

3.1 Introduction

Microbial iron reduction (MIR) is an important and ubiquitous natural process in the biogeochemical cycling of iron and the oxidation of organic matter in anaerobic sedimentary and subsurface environments [168]. Although non-enzymatic reduction of Fe^{3+} under anaerobic conditions can occur [169], dissimilatory MIR by iron-reducing bacteria (IRBs), which use Fe^{3+} as the terminal electron acceptor in respiration, is considered the most important mechanism for converting Fe^{3+} to Fe^{2+} [168, 170]. During the MIR process, IRBs can oxidize and mineralize a large variety of organic compounds and produce CO_2 [73-76], which plays a major role in carbon cycling in anaerobic environments [168]. Since Fe^{3+} substrates are usually insoluble, IRBs and mixed MIR communities have evolved various strategies to transfer electrons, either through direct contact [171] or via intermediate electron carriers [172, 173], to solid Fe^{3+} substrates. Since the initial isolation of *Shewanella* and *Geobacter* species [67, 174], our knowledge about the phylogenetic diversity of IRBs has greatly expanded to include many species across the domain Bacteria [175, 176], further reflecting the ubiquity of the MIR process in the environment.

The metabolic capability of the MIR process has been extensively explored for biotechnology applications, including using Fe^{3+} to enhance the bioremediation of organic pollutants [168] and using the MIR communities to transfer electrons to external anodes for energy harvesting in microbial fuel cells [177]. However, the possibility of using MIR process in water purification, particularly in bacterial pathogen inactivation and removal, has not been investigated. Although anaerobic environments in general favor bacterial pathogen survival, the MIR process contains several features that may

enhance pathogen inactivation. First of all, amorphous ferric oxides are known to be strong adsorbent for bacterial cells [45, 140], which could concentrate bacterial cells to surfaces where MIR occurs. Second, the MIR process produces Fe^{2+} as a metabolic waste, which was recently shown to be a powerful bactericidal agent under anoxic conditions [178-180]. Thirdly, the metabolic diversity of IRBs and mixed MIR communities in degrading a large variety of organic substrates [73-76] indicates that cellular materials of inactivated fecal bacteria cells may be used as carbon and energy sources, which is supported by *Shewanella*'s capability of consuming extracellular DNA [181].

Therefore, the objectives of this study were (1) to determine if MIR process can enhance the inactivation of the model organism *Escherichia coli* under anaerobic conditions and (2) to identify potential inactivation mechanisms. Laboratory microcosms were established to compare the inactivation of *E. coli* cells in the presence/absence of MIR activity and between the MIR condition and other anaerobic redox conditions. *E. coli* inactivation in the presence of different Fe^{2+} concentrations was quantified to verify the bactericidal effect of Fe^{2+} under anoxic condition. The capability of the MIR process to use *E. coli* cells as the sole electron source for energy metabolism was also investigated.

3.2 Materials and Methods

3.2.1 Bacterial strains, cultivation, and enumeration

E. coli ATCC 29522 was used as the model fecal bacterium in this study. A single fresh colony from overnight growth on an LB agar plate was used to inoculate LB broth,

which was cultivated at 37°C with constant shaking at 200 rpm. Stationary phase cells ($OD_{600} > 1.2$) were collected by centrifugation at 10,000 x g for 3 minutes. The cell pellet was washed by resuspending in 0.1x PBS buffer (pH 7.2) and then pelleting by centrifugation for five times to remove residual broth nutrients. The cell pellet was subsequently resuspended in sterile artificial freshwater medium (see below for composition) to prepare cell stock solution with a target OD_{600} of 0.8, which contained approximately 10^9 - 10^{10} CFU/mL, and was used in subsequent inactivation experiments. *E. coli* in the stock solution and samples collected from the experiments was enumerated by spread plating of appropriate 10-fold sequential dilutions on the mTEC agar [182].

3.2.2 Iron-coated sand preparation

Quartz sand was coated by amorphous ferric oxyhydroxide (FeOOH) following the procedure described by Mills *et al.* [140]. Sand was heated at 550°C for 3 hours and then rinsed several times with DI water to remove organic matters. Trace metal was washed by soaking the sand in concentrated 10 M HCl for 24 hours, then rinsing in 0.01 M NaOH, and finally rinsed with DI water until the effluent pH reached 8.0 ± 0.1 . Sand was dried at 110°C, and then stored in a clean bottle for later use. The cleaned dry quartz sand was immersed in 400 mL of FeCl₃ solution (50 g/L of FeCl₃.H₂O, pH 1.9), and 30 mL of NaOH (0.5 M) was added instantaneously followed by gradual addition of 1mL NaOH (0.5 M) until pH reaches 4.5-5.0. The mixture was then shaken for 36 hours to allow further coating of FeOOH onto sand surfaces. Iron coated sand was then rinsed with DI water, air dried, and saved in a clean bottle for later use.

3.2.3 Microbial Inoculum preparation

Anaerobic microbial inocula were first enriched using FeOOH, SO_4^{2-} , and NO_3^- as the terminal electron acceptor for iron-reducing bacteria (IRBs), sulfate-reducing bacteria (SRBs), and denitrifiers. Artificial freshwater medium used in the enrichment contained 2.5 g/L NaHCO_3 , 0.1 g/L NaCl, 0.1 g/L NaCl, 0.1 g/L KCl, 0.1 g/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.5 g/L NH_4Cl , 0.6 g/L NaH_2PO_4 , 0.005 g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.001 g/L NaMoO_4 , and 0.05 g/L yeast extract [67]. The enrichments were established in 160 mL serum bottles, containing 100 mL of artificial freshwater medium and 10 mM of sodium acetate as the electron donor. The microcosm for IRB enrichment contained 20 g of FeOOH-coated quartz sand, the microcosm for SRB enrichment contained 10mM of MgSO_4 , and the microcosm for denitrifiers contained 10 mM MgNO_3 . The microcosms were inoculated with 1.0 g of anaerobic sediment sample collected from a pond near the Waipahu Stream where iron-rich Haplustoll soil is present. The serum bottles were capped with rubble stopper and sealed with aluminum crimp. Air in the head space of serum bottles was removed by vacuum and followed by 15 minutes N_2 bubbling for three times. The microcosms was incubated at room temperature in dark, and the enrichment of IRBs, SRBs, and denitrifiers were verified by Fe^{2+} accumulation, SO_4^{2-} concentration reduction, and NO_3^- concentration reduction, respectively (data not shown). To pool the three inocula into one anaerobic inoculum, the microcosms were thoroughly shaken by hand for three minutes, and 10 mL of each suspension was withdrawn with a syringe and injected into an anoxic serum bottle. The microbial community of the pooled anaerobic inoculum was determined by Illumina sequencing of 16S rRNA gene following the

procedure described by Zhang *et al.* (2015). The microbial community contained common IRBs, SRBs, and denitrifiers (Table 3.1).

Table 3.1. Phylogenetic affiliation of OTUs with a relative abundance (RA) larger than 5% in the pooled anaerobic inocula, and their putative function based literature inference.

OTUs	Taxon ^a	RA (%)	Putative function	Ref.
1	<i>Erysipelotrichaceae</i> (f)	20.9	--	--
2	<i>Fusibacter</i>	9.3	--	--
3	<i>Geobacter</i>	5.7	IRB	[183]
4	<i>Bacteroidales</i> (o)	5.2	--	--
5	<i>Comamonadaceae</i> (f)	4.1	--	--
6	<i>Azospira</i>	3.8	Denitrifier	[184]
7	<i>Azoarcus</i>	3.7	--	--
8	<i>Pelobacteraceae</i> (f)	2.8	--	--
9	<i>Betaproteobacteria</i> (c)	2.5	--	--
10	<i>Bacteria</i> (k)	2.5	--	--
11	<i>Desulfobulbaceae</i> (f)	2.3	SRB	[185]
12	<i>Bacteroidales</i> (o)	1.9	--	--
13	<i>Thauera</i>	1.8	--	--
14	<i>Dechloromonas</i>	1.8	--	--
15	<i>Treponema</i>	1.6	--	--
16	<i>Bacteroidetes</i> (p)	1.5	--	--
17	<i>Desulfuromonadales</i> (o)	1.2	--	--
18	<i>Acholeplasma</i>	1.0	--	--
19	<i>Spirochaetes</i> (p)	1.0	--	--
20	<i>Candidatus Solibacter</i>	0.9	--	--
21	<i>Rhodocyclaceae</i> (f)	0.9	--	--
22	<i>Methanosarcina</i>	0.9	Methanogen	[186]
23	<i>Syntrophobacter</i>	0.8	--	--
24	<i>Proteobacteria</i> (p)	0.8	--	--
25	<i>Ruminococcaceae</i> (f)	0.7	--	--
26	<i>Clostridiaceae</i> (f)	0.6	--	--
27	<i>Cyclobacteriaceae</i> (f)	0.6	--	--
28	<i>Pedosphaerales</i> (o)	0.5	--	--

^a The taxon of the OTUs was by default resolved at the genus level. The OTUs taxon followed by (k), (p), (c), (o), and (f) only found matches in the database at kingdom, phylum, class, order, and family levels, respectively.

3.2.4 Microcosm setup with different redox conditions

Anaerobic microcosms to determine the decay kinetics of *E. coli* cells under different redox conditions were established in the same way as the inoculum enrichment described above. Four sets of microcosms, each containing three independent microcosms as biological replicates, were established. Two sets of identical microcosms contained FeOOH-coated quartz sand, with one set receiving the anaerobic inoculum (termed active MIR microcosms) and the other set receiving no inoculum and hence remaining sterile (control microcosms). The active MIR microcosm and the control microcosms were used to compare the effect of active MIR activity on *E. coli* inactivation. The microcosms receiving sulfate were to establish microbial sulfate reduction (MSR) condition, while the microcosms receiving nitrate were to establish microbial nitrate reduction condition (MNR). The MSR and MNR microcosms were used to compare with the *E. coli* inactivation in the MIR microcosms. The pooled anaerobic inoculum (1mL) was injected into the MIR, MSR, and MDN microcosms using a syringe. An equal amount of *E. coli* cells (ca. 3×10^9 CFU) was also injected into all microcosms using freshly prepared *E. coli* stock solutions. The microcosms were then incubated at room temperature in dark without shaking. Samples were collected daily by first vigorously shaking serum bottles for 2 minutes followed by immediately withdrawing 1mL of the mixtures using a syringe.

3.2.5 Inactivation of *E. coli* by Fe²⁺

The impact of different Fe²⁺ concentrations on the inactivation of *E. coli* was investigated in 50 mL serum bottles under anoxic condition. Each bottle contained 30 mL

of 0.1 x PBS buffer that was deoxygenated by flushing N₂ into the headspace for 15 minutes before sealed off with a rubber stopper and aluminum seal. *E. coli* cells from the stock solution were injected into the bottles to reach an initial cell concentration of approximately 10⁷ CFU/mL. Different final concentrations of Fe²⁺ (0, 0.05, 0.1, 0.2, and 0.5 mM) were added into the bottles to make the experimental treatments. Each treatment used three bottles as biological replicates. The bottles were incubated under the anoxic condition at room temperature (22 ± 0.5°C) on a shaker at 40 rpm. Samples were taken from each bottle (1.0 mL) after fully mixing at different incubation time (0, 1, 12, 36, and 60 hours). The samples were subjected to 10-fold serial dilution in sterile 0.1 x PBS buffer, and the culturable *E. coli* cells were enumerated as described above.

3.2.6 Carbon source experiment

Three sets of MIR microcosms, each in triplicate, were also established to test if *E. coli* cells could be used as the sole carbon source for MIR. The basic microcosms setup was the same as described above, except for the carbon source. The microcosms either received sodium acetate (final concentration 0.2 mM), *E. coli* cells (ca. 10¹⁰ CFU), or no carbon source (control). The carbon source equivalency of the spiked *E. coli* cells was determined by first autoclaving the samples at 121°C for 20 min to lyse the cells, and then analyzing the cell lysates by TOC analysis (described below), which gave an average TOC of 232.6 mg C/L. The acetate concentration used (0.2 mM) gives a theoretical TOC of 4.8 mg C/L, and the measured TOC concentration was 2.7±0.3 mg C/L. The microcosms were inoculated by injecting 1 mL of the anaerobic inoculum using a syringe. All microcosms were incubated at room temperature, in dark, and without

shaking. The microcosms were sampled daily using the same procedure described above, and the samples were analyzed for Fe²⁺ and TOC.

3.2.7 Chemical analysis

Fe²⁺ was measured using the colorimetric ferrozine assay following the procedure described by Lovley *et al.* [65]. Briefly, liquid samples (0.1mL) were transferred into 5 ml of ferrozine (1 g/liter) in 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (pH 7). The mixtures were vortexed for 15 s and then filtered through a Nuclepore filter (0.2 µm). Filtrates were measured for light absorbance at wavelength 562 nm using a spectrometer (Hach DR/4000U), and Fe²⁺ concentration was calculated based on standard curves established by using ferrous ethylene-diammonium sulfate as standard solutions. Anions (SO₄²⁻ and NO₃⁻) were quantified by using a Dionex ICS-1100 Ion Chromatograph equipped with a 4 mm AS14A analytical column. 25µL of samples was injected into the analytical system by an AS-DV auto-sampler utilizing Dionex filter cap vials that automatically filter the samples before loading into injection loop. TOC concentration in the samples was determined by using a TOC analyzer (Shimadzu).

3.2.8 Data analysis

The Fe²⁺ concentration difference between the sterile control microcosms and active MIR microcosms was tested using *t*-test for individual sampling dates. The Fe²⁺ production rate during the three sequential periods following repetitive *E. coli* cell spikes was determined by linear regression of Fe²⁺ concentration data versus time. *E. coli* cell inactivation was modeled using the 1st order model ($\ln\left(\frac{C_t}{C_0}\right) = -k_d t$), where C_t and C₀ are

the concentrations of *E. coli* cells in the microcosms at time t and time zero, respectively. k_d is the decay coefficient of the 1st-order decay model and was identified through linear regression of natural log transformed concentration data. Comparison of *E. coli* inactivation rates was performed using ANCOVA with Tukey's post-hoc test. ANOVA was used to test if there was difference in *E. coli* concentration in the Fe²⁺ inactivation experiment, and to test if Fe²⁺ and TOC concentrations were different among the different carbon source treatments. Statistical tests were conducted either in the Microsoft Excel with a statistiXL plug-in or using Sigma 10.0, and the default significance level is 0.05, unless stated otherwise.

3.3 Results

3.3.1 *E. coli* inactivation in the presence of MIR

The impact of MIR on the inactivation of *E. coli* cells was investigated by comparing the decay patterns of *E. coli* cells in the active MIR microcosms, which received the anaerobic inoculum, to those in the control microcosms, which did not receive the anaerobic inoculum. During the incubation, Fe²⁺ concentration in the active MIR microcosms continued to increase over time, and became significantly higher than that in the control microcosms after Day 1 (t test, $P < 0.05$), indicating successful development of MIR activity (Figure 3.1A). The Fe²⁺ production rates were calculated to be 0.65, 1.20, and 5.78 $\mu\text{mol}/(\text{L}\cdot\text{day})$ for the three periods following the three repetitive spikes of *E. coli* cells on Days 0, 13, and 25, respectively. The Fe²⁺ production rate in the second period was higher than that in the first period (although not statistically significant), the Fe²⁺ production rate was significantly higher the third period than in the

first two periods (ANCOVA, $P < 0.001$), indicating continuous and significant increase in MIR activity over time.

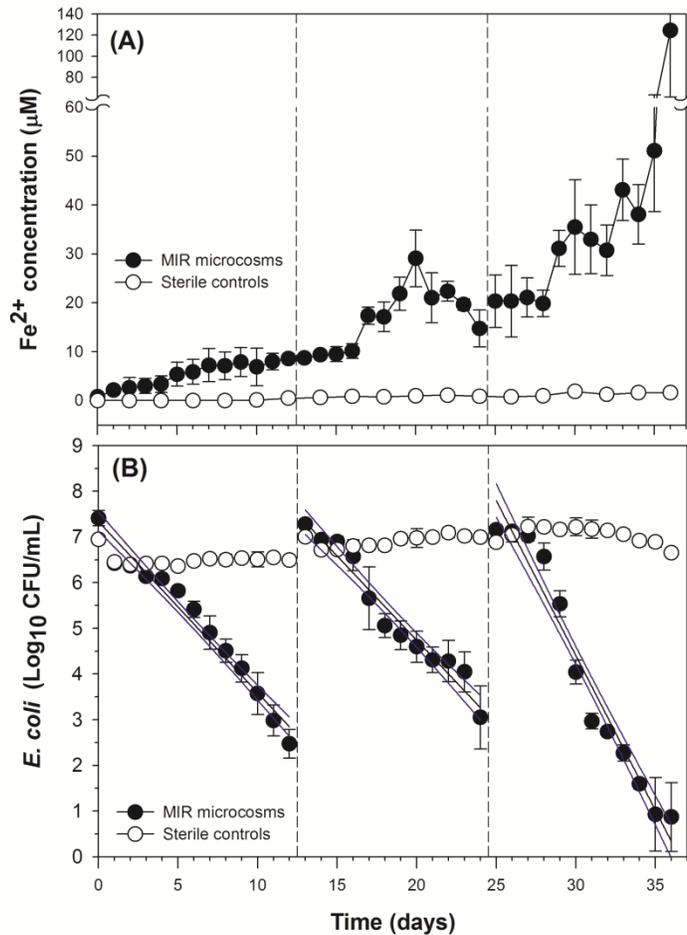


Figure 3.1. Production of Fe²⁺ (A) and inactivation of *E. coli* cells (B) in the active MIR and control microcosms. Repetitive spike of *E. coli* cells (c.a. 10⁷ CFU/mL) occurred on Days 0, 12, and 24, as indicated by the dashed lines. Error bars indicate the standard deviation of the mean of triplicate microcosms.

Significantly faster inactivation of *E. coli* cells was observed in the active MIR microcosms than in the control microcosms following all three repetitive spikes of *E. coli* cells (Figure 3.1B). The control microcosms exhibited negligible *E. coli* inactivation during the experimental course. In the active MIR microcosms, *E. coli* exhibited first-order inactivation rates of 0.86, 0.85, and 1.56 day⁻¹ in the three periods following the three repetitive spikes, respectively (Table 3.2). Significant difference was detected

amongst the three decay rates, and Tukey's post-hoc tests showed that there was no significant difference in the inactivation rates during the first two periods (0.86 and 0.85 day⁻¹), inactivation rate after the third spike (1.56 day⁻¹) was significantly higher than those observed after the first two periods (ANCOVA, P<0.001). The increasingly higher *E. coli* inactivation rates corresponded well to the increasingly higher MIR activity over the experimental course.

Table 3.2. Average inactivation rates of *E. coli* cells in microcosms under different redox conditions (k_{MIR} , k_{MNR} , and k_{MSR}), goodness-of-fit of the linear regression (r^2), and rate comparisons *.

Spike	k_{MIR}	r^2	k_{MNR}	r^2	k_{MSR}	r^2	$k_{MIR} > k_{MNR}$	$k_{MIR} > k_{MSR}$
1	0.86±0.03	0.95	0.74±0.04	0.88	0.24±0.02	0.78	P=0.04	P<0.001
2	0.85±0.05	0.91	0.45±0.05	0.73	0.27±0.03	0.65	P<0.001	P<0.001
3	1.56±0.06	0.95	0.87±0.06	0.84	0.60±0.04	0.89	P<0.001	P<0.001

* ANCOVA with Tukey's post hoc test.

3.3.2 Comparison with other anaerobic redox conditions

To further test the impact of MIR activity on *E. coli* inactivation, the MIR microcosms were also compared with the MSR and MNR microcosms, which had the same microcosm setup as the MIR microcosms, received the same anaerobic inoculum, but were provided with SO₄²⁻ or NO₃⁻, respectively, to establish different anaerobic redox conditions. Microbial sulfate reduction and nitrate reduction were developed in the MSR and MNR microcosms, respectively, as indicated by the gradual decrease of SO₄²⁻ and NO₃⁻ concentration over time (Figure 3.2). The different microcosms exhibited different *E. coli* inactivation patterns, with the MIR microcosms showing the fastest inactivation after all three repetitive spikes (Figure 3.3). Fitting of the *E. coli* inactivation data to the first order model gave inactivation rates of 0.45-0.87 day⁻¹ and 0.24-0.60 day⁻¹ for the MNR and MSR microcosms, respectively, which were significantly smaller than the

those in the MIR microcosms after all three repetitive spikes of *E. coli* cells (ANCOVA, $P \leq 0.04$) (Table 3.2).

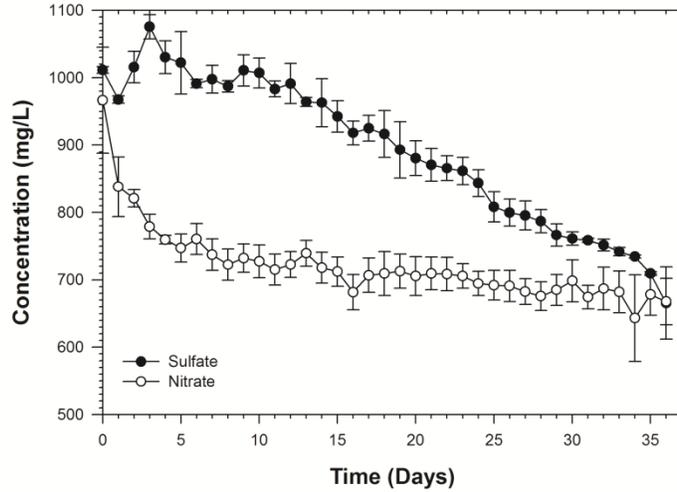


Figure 3.2. Reduction of sulfate and nitrate in the MSR and MDN microcosms over time. The error bars indicate the standard deviation of the mean of triplicate microcosms

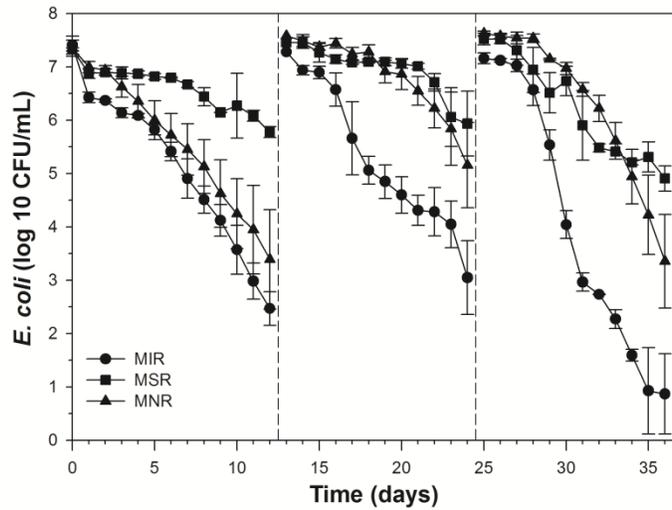


Figure 3.3. Inactivation of *E. coli* cells under different redox conditions (MIR, MSR, and MNR). Repetitive spike of *E. coli* cells (c.a. 10^7 CFU/mL) occurred on Days 0, 12, and 24, as indicated by the dashed lines. Error bars indicate the standard deviation of the mean of triplicate microcosms.

3.3.3 Inactivation of *E. coli* cells by ferrous ion

The bactericidal effect of Fe^{2+} produced from the MIR activity was investigated by determining *E. coli* inactivation rates in the presence of different Fe^{2+} concentrations under anoxic condition (Figure 3.4A). *E. coli* inactivation was negligible in the absence of Fe^{2+} over the entire 60 hours of incubation, while significantly lower *E. coli* concentrations were observed in the presence of Fe^{2+} within the first hour of incubation (ANOVA, $P < 0.01$). Since the majority of *E. coli* inactivation occurred within the first hour and then tailed off almost completely, the initial *E. coli* inactivation rates were calculated using the inactivation data within the first hour (Figure 3.4B). Higher Fe^{2+} concentrations corresponded to larger *E. coli* inactivation rates; the microcosms receiving 0, 0.05, 0.1, 0.2, and 0.5 mM Fe^{2+} exhibited an initial decay rates of 0.001, 0.44, 0.81, 2.5, and 6.1 hour^{-1} , respectively. The observed *E. coli* inactivation rates showed a linear relationship to Fe^{2+} concentrations, as indicated by the excellent goodness-of-fit ($r^2 = 0.99$).

3.3.4 *E. coli* cells as sole electron source

The capability of MIR to use *E. coli* cells as the sole electron source was determined by comparing Fe^{2+} production in MIR microcosms that received *E. coli* cells (2.8×10^{10} CFU/ml) to two sets of control microcosms that received either 0.2 mM either acetate (positive control) or no carbon source (negative control) (Figure 3.5A). On Day 0, there was no significant difference in Fe^{2+} concentration amongst all microcosms (ANOVA, $P = 0.99$). Starting from Day 1 to Day 8, the positive control microcosms showed a Fe^{2+} concentration range of 50.6 to 75.7 μM , which were significantly higher than that in the negative controls (ANOVA, $P < 0.001$), indicating the development of

active MIR activity in the microcosms. The negative control microcosms showed very low Fe^{2+} concentrations and negligible fluctuation over the eight-day experimental course (concentration range: 2.6-15.4 μM).

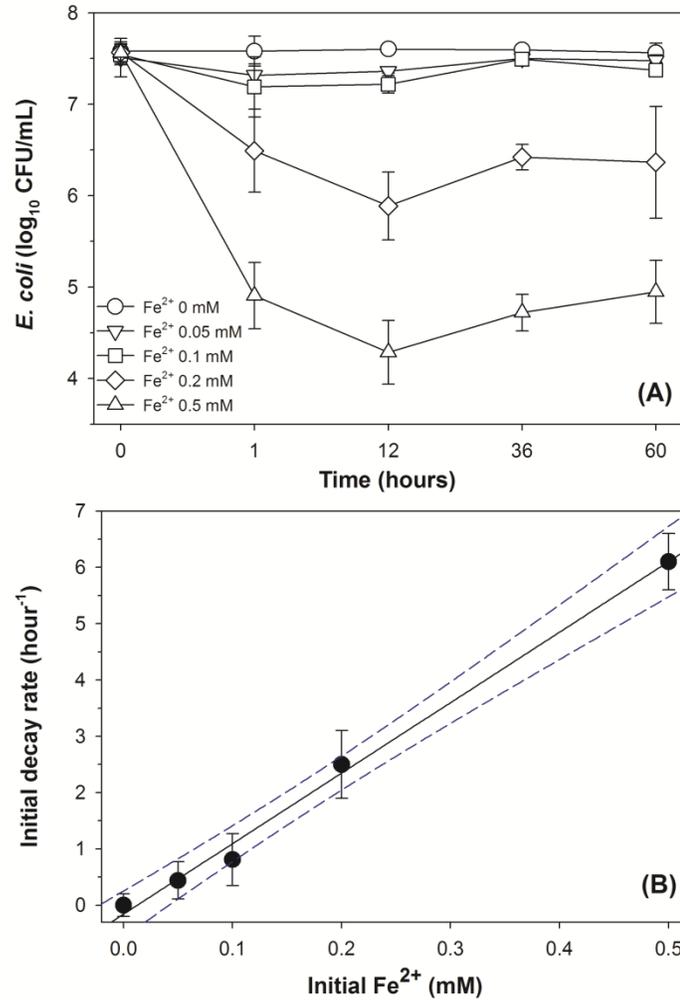


Figure 3.4. Inactivation of *E. coli* under different Fe^{2+} concentration over different exposure times (A), and linear regression between the first-hour inactivation rate and Fe^{2+} concentration (B). Error bars indicate the standard deviation of the mean of triplicate microcosms. The dashed lines in compartment B represent 95% confidence bands.

Starting from Day 1, the microcosms that received *E. coli* cells as the sole carbon source showed significantly higher concentrations of Fe^{2+} than both sets of control

microcosms (ANOVA, $P < 0.001$). The Fe^{2+} concentration continued to increase over time, reached $995.2 \pm 26.1 \mu\text{M}$ on Day 6, and then gradually tailed off. Correspondingly, initial TOC concentrations in the MIR microcosms that received *E. coli* cells was also significantly higher than the two sets of control microcosms, and its decreasing pattern over time, which tailed off after Day 6, coincided with the Fe^{2+} concentration increase pattern.

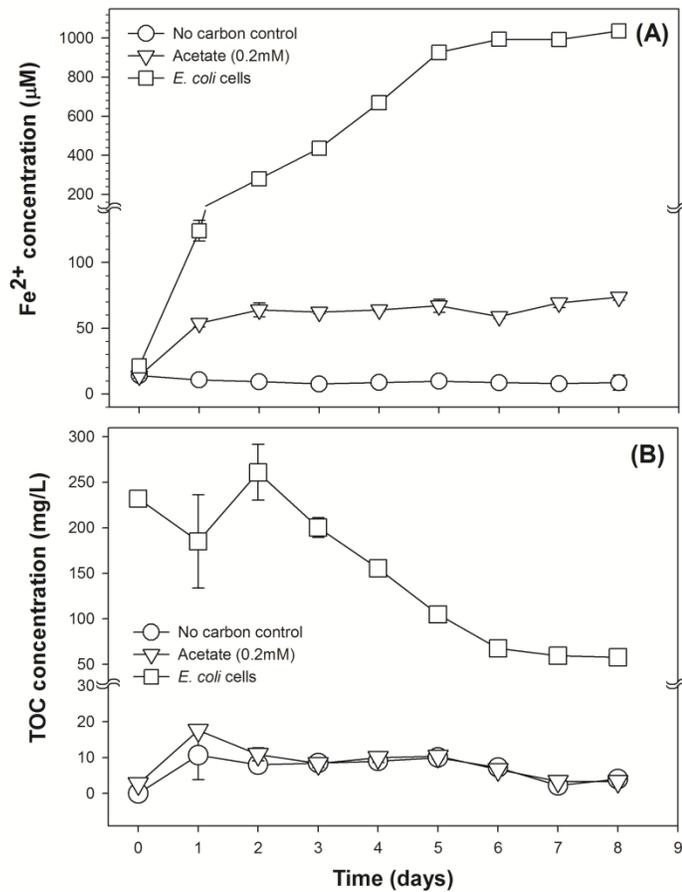


Figure 3.5. Fe^{2+} concentrations in MIR microcosms that received no carbon, acetate or *E. coli* cells as the sole electron source (A), and their corresponding TOC concentration change over time (B). Error bars indicate the standard deviation of the mean of triplicate microcosms.

3.4 Discussions

3.4.1 *E. coli* inactivation by the presence of MIR

The impact of MIR activity on the inactivation of *E. coli* cells was clearly demonstrated by the significantly faster decay in the active MIR microcosms than in the sterile control microcosms, which was repetitively observed over the three sequential spikes of *E. coli* cells (Figure 3.1B and Table 3.2). Since the only difference between the active MIR and the control microcosms was the anaerobic inoculum, the observed difference in decay patterns should be primarily attributed to the presence of the anaerobic inoculum and resulting MIR activity in the active MIR microcosms. The limited *E. coli* concentration reduction over time in the control microcosms indicates that abiotic factors, such as cell recovery from the iron coated surface [45, 140] and abiotic inactivation by iron oxides surface [52], made limited contribution to the reduction of culturable *E. coli* cell number under the experimental conditions. Endogenous decay of *E. coli* cells under sterile anaerobic conditions is usually very slow [187], which is in line with the slow *E. coli* inactivation observed in the sterile control microcosms.

The correspondence between higher MIR activity and faster inactivation rate (Figure 3.1) and the faster inactivation under the MIR condition than under the MNR and MSR redox conditions (Figure 3.3) provided further support to the association between MIR activity and enhanced *E. coli* inactivation. In the active MIR microcosm, increasingly higher Fe^{2+} production rates over time indicate continuous growth of iron-reducing bacteria and higher MIR activity in the third period than in the first two (Figure 3.1B). This is in agreement with the slow growth of IRBs; for example, the growth rate of *Geobacter metallireducens* was reported to be below 0.003 hour^{-1} [188].

3.4.2 Comparison with other anaerobic redox conditions

Comparing *E. coli* inactivation in the MIR microcosms to that in the MNR and MSR microcosms provided further support to the superiority of MIR on *E. coli* inactivation. The MIR, MNR, and MSR microcosms started with the same anaerobic inoculum, and the development of respective anaerobic activities followed the normal expectation of typical growth rates of the individual anaerobic organisms (i.e., MNR>MSR>MIR). Although during the first spike, there was no significant difference in inactivation rates between the MIR and MNR microcosms, significantly faster inactivation of *E. coli* cells was observed in the MIR microcosms than the other microcosms during both the second and third spikes. The increasingly larger difference in decay rates between the MIR microcosms and other microcosms as time progressed corresponded well to its slower growth rate in comparison to MNR and MSR, suggesting that even faster inactivation could be achievable with higher MIR activity.

The observation of significantly faster *E. coli* inactivation in the presence of MIR activities highlights the importance of biotic stresses to the inactivation of fecal bacteria. Several recent studies have shown that *E. coli* often exhibit significantly faster decay rates in the presence of indigenous microbiota in soil [189, 190], freshwater [189, 190], seawater [191], and beach sand [187]. Different types of biotic stresses, including protozoa predation [192, 193], phage infection [194, 195] and bacterial competition [187, 196-199], have been shown to play significant roles in *E. coli* inactivation in the environment. The significant difference in *E. coli* inactivation between the different redox conditions indicates that bacterial competition from the MIR community played a significant role in the observed *E. coli* cell inactivation.

Bacterial interspecies competition can involve many different mechanisms, such as nutrient competition and antibiotic production [200]. Although bacterial metabolic wastes are generally considered to be adverse to the waste producer themselves, their impact on other bacterial populations, particular those allochthonous to the prevailing microbial community, has not been explicitly studied. Previous works have shown that Fe^{2+} can inactivate *E. coli* cells under anoxic conditions [178-180], and results in this study suggest that the Fe^{2+} produced as a metabolic waste of MIR can rapidly inactivate *E. coli* cells (Figure 3.4) and hence may function as a mechanism in bacterial interspecies competition. Since many soil bacteria can produce secondary metabolites (antibiotics) to participate in inter-species competition [201], it is not totally surprising bacterial metabolic wastes may also fulfill similar ecological functions.

3.4.3 Inactivation of *E. coli* by ferrous iron

Regarding to the chemical mechanism underlying the inactivation, previous authors suggested that Fe^{2+} could have reacted with intracellular H_2O_2 to produce reactive oxygen species (such as hydroxyl radical) via the Fenton's reaction [178, 180], which are strong oxidants with bactericidal effects [202]. Aerobically-grown *E. coli* cells typically generates 14 μM of H_2O_2 per second [107] and maintain a steady-state 0.1-0.2 μM of H_2O_2 due to various scavenging mechanisms [203]. Although the intracellular H_2O_2 produced under aerobic condition could have persisted and been carried over into the anoxic condition used in this study, the linear dependency of *E. coli* inactivation on Fe^{2+} concentration observed here and in previous studies [178-180] suggest Fe^{2+} , rather than intracellular H_2O_2 , was the limiting factor, hence partially dissuading the contribution

from Fenton's reaction. Further research is needed to fully elucidate the inactivation mechanism(s).

3.4.4 *E. coli* cells as sole electron source

The inactivated *E. coli* cells were used as the sole electron source in the MIR process, as indicated by the increase in Fe^{2+} concentration and concurrent reduction of TOC in the microcosms (Figure 3.5). Individual IRBs are capable of using a wide variety of organic substrates as electron source for respiratory energy generation, including short chain fatty acids [74, 204], low molecular weight petroleum organics [73, 75, 76, 90, 91], aromatic compounds [73-76], and even extracellular DNA [181]. With the assistance of other members in the complex MIR community, some of which may be equipped to degrade other bacterial cellular components [197], the MIR community could efficiently degrade the inactivated *E. coli* cells and couple that to dissimilatory iron reduction. This coupling potentially provides an ecological impetus for the MIR community to inactivate exogenous *E. coli* cells, providing a positive feedback loop as more Fe^{2+} inactivates more *E. coli* cells, which leads to higher MIR activity and higher Fe^{2+} .

3.5 Conclusions

Since MIR is an important biogeochemical process in sedimentary and subsurface environments and is known to enhance the biodegradation of a large variety of organic pollutants, MIR is expected to influence water quality in such environments [176]. Results from this study, for the first time, demonstrated that the MIR activity can also significantly enhance the inactivation of *E. coli*, and by inference other fecal bacteria. The production of Fe^{2+} as the metabolic waste of MIR was identified as a mechanisms in

E. coli inactivation under the anaerobic condition. Since the inactivated *E. coli* cells were shown to be used by the MIR community as an electron source to drive Fe^{3+} reduction, this represents a new mechanism for bacterial inter-species competition. This knowledge could further improve our understanding of the fate of fecal bacteria in natural sedimentary and subsurface environments where the MIR process is prevalent, and may also be explored for the enhancement of pathogen removal in many engineering processes, such as storm water bioretention facilities, aquifer artificial recharge, and low-cost soil based water reclamation.

CHAPTER 4. REMOVAL OF TOTAL ORGANIC CARBON AND FECAL BACTERIA IN AN ANAEROBIC-AEROBIC TWO STAGE FILTER

Abstract

Pathogenic bacteria are high concerns in water reclamation. Conventional treatment methods used to remove the microbial contaminants are energy intensive and remain concerns of byproducts. Sand filtration is a low-cost technology that has been employed for secondary disinfection in reclaimed water. However, limitations of slow sand filtration depend on operation and configuration. This study aims to explore anaerobic-aerobic sand filtration for enhancement of removal of pathogenic bacteria. Removal mechanism was proposed due to the interaction of bacteria with iron coated sand and biofilm. Precipitation and adsorption of bacteria to iron hydroxide sludge will clean the reclaimed water prior to discharge to receiving environment. Results showed that removal of total organic carbon (TOC) reached 70-95 %, and remaining concentration was 1.7-7.85 mg/L. High removal rates were obtained for *Escherichia coli*, *Enterococcus faecalis*, and *Salmonella* Typhimurium. The results demonstrated that iron coated sand column efficiently removed fecal bacteria and pathogens in artificial secondary wastewater effluent. Anaerobic condition naturally established in filtration media provided favor reducing environment for ferric iron reduction in couple with oxidation of organic substrates. The ferrous production from the anaerobic iron reduction was oxidized to form ferric oxides in the aerobic filter and led to the further removal of fecal bacteria and pathogens via precipitation and adsorption to iron sludge. The

anaerobic-aerobic two-stage filtration system may be applied to improve the quality of reclaimed water.

4.1 Introduction

Technical and economical feasibilities are the trend of wastewater reclamation for future sustainable development. While freshwater sources are limited due to drought and pollution, treated wastewater is primarily accepted as a water source for some major applications including landscape or agricultural irrigation, groundwater recharge, surface water replenishment, and toilet flushing. In general, conventional wastewater treatment processes for water reclamation comprise physical, chemical, and biological mechanisms which effectively remove suspended solid, assimilate organic substrates, taste, odor issues, and some levels of pathogenic microorganisms. However, it was reported that pathogenic bacteria and virus were not adequately removed from reclaimed water by traditional disinfection methods [6, 8]. Advanced treatment methods such as nano-filtration (NF), reverse osmosis (RO), advanced oxidation, and activated carbon filtration effectively removed micro-pollutants to produced high quality reclaimed water [14-16, 205]; however, the cost of these technologies remained relative high due to energy intensive [12, 205]. Traditional disinfection methods could be costly and bear additional health risks such as disinfection byproducts [11-13]. In this study, an inexpensive biofilter is investigated as a tertiary treatment method for the secondary effluent wastewater to improve the quality and reduce the pricing of water reclamation.

Slow sand filter (SSF) has become an attractive technology for wastewater reclamation due to its simplicity in configuration and low cost in operation and maintenance. It had been investigated to remove suspended solids (SS), organic substrates, and pathogens in effluents of secondary and anaerobic treatments for irrigation

applications [25, 38, 206, 207]. System configurations and operational conditions were important factors in biosand filters and strongly affected the removal of bacteria in feeding water [25, 30, 207]. It was suggested that the design of surface areas of porous media in SSF must be larger than $2000\text{m}^2/\text{m}^2$ in order to remove bacteria [25]. Previous studies addressed that predation, straining, and adsorption were the removal mechanisms of bacteria removal in old fashion sand filtration [27, 30-32]. However, bacteria were effectively removed only in the top portion of filter media (0-2 cm) where the biomass development occurred [25, 30, 36]. Activities of microorganism in the upper portion of filtration media led to the decreasing of oxygen concentration which resulted in anoxic to anaerobic zone where denitrification occurred [78].

A new approach in sand filtration that has been recently increasing attention to researchers in the field to improve removal efficiencies, as well as a broad range of contaminants, is the surface modification of filtration media by metallic oxides. Iron oxides in the granular forms, iron-based materials, or metallic coating layers to porous media have been examined as a potential absorbent to organic and inorganic contaminants in freshwater and wastewater. Previous studies showed that iron oxide coated sand filters resulted in strong and irreversible absorption of bacteria to media surfaces [45, 140]. Due to its positive charge at neutral pH, metallic oxides coating media appear to have advantages in removing negative particulate such as microorganisms in comparison to quartz sand in filtration systems. It was reported that heavy metals and natural organic compounds were also absorbed to iron coated porous media in filtration due to physical and chemical interactions mechanisms [47, 49, 51, 208, 209]. Microbial community developing in old fashion sand filtration system has been addressed to play an

important role in removing microorganisms in water sources. However, availability of electron donors and acceptors led to the selective establishment of microbial communities at low portion of biofilters [34]. Metallic contents of solid media showed an advantage to enhance the colonization and microbial activities in filtration process [54, 210]. In addition, ferric iron also served as an electron acceptor for anaerobic respiration to control odor in swine wastewater [211]. It was revealed that iron reducing bacteria required direct contact for growth [79, 80] and potentially reproduced in sufficient energy for respiration [212]. However, limited information is available about using iron oxide coated sand media as an electron acceptor in a SSF system to promote microbial activities for increasingly removing microbial contaminants and organic substrates.

Although the occurrence of iron reduction coupled with the oxidation of organic substrates has been widely found in the subsurface and sedimentary environments [213], microbial reduction of ferric iron in a column study is not fully understood. In addition, using ferric oxide as an electron acceptor for anaerobic respiration has not been recognized yet as a means to facilitate the oxidation of biodegradable organic compounds in wastewater treatment systems. Anoxic and anaerobic zones in the slow sand filter were addressed to be analogous to the sedimentary environment [78] which promotes a favor environmental condition for MIR. When sand media is coated by iron oxides, iron reducing bacteria will be probably predominant in anoxic and anaerobic microenvironments. Roden et al. (2000) reported that a continuous flow system increased the MIR and associated with bacterial growth because the effects of surface-bound ferrous on oxide reduction activity was decreased [214]. Development of biofilm under anaerobic condition may be enhanced by contact growth of IRB [79, 80] and results

positive effects on performances of the filtration systems in addition to physicochemical mechanism in SSF. A previous study showed that IRB were active to reduce uranium under the anaerobic condition in filter columns [215]. Nevertheless, no effort has been made to assess the effects of iron reducing bacteria at lower depth filter column for wastewater reclamation.

This study was the first to investigate the cost effective technology using anaerobic-aerobic sand filter for wastewater reclamation. Recent observations on water reclamation showed that pathogenic bacteria were highly detected in treated water [6, 8] and pose a potential health risk when using reclaimed water for agricultural irrigation [7, 216]. The goals of this study were to treat artificial secondary effluents containing fecal bacteria and pathogens to reclaimed water in two-stages of sand filtration processes. It was hypothesized that anaerobic iron coated sand filter promote a higher volume of biomass than the old-fashion sand filter, which results in biological ripening for removing bacteria as well as TOC in secondary effluent wastewater.

4.2 Materials and methods

4.2.1 Bacteria selection and enumeration

Nonpathogenic *Escherichia coli* ATCC 29522, pathogenic *Enterococci faecalis* ATCC 29212 (*E. faecalis*) and *Salmonella* Typhimurium (*S. Typhimurium*) were used as testing bacteria in this study. *E. coli* was selected to present gram-negative and well-characterized that been widely studied in bacterial transport through porous media. *E. faecalis* was chosen to present the gram-positive bacteria. *S. Typhimurium* was selected as a representative pathogenic bacterium widely found in wastewater. Experimental

bacteria were obtained from growing of single fresh colony in 20 mL Luria-Bertani (LB) broth at 37°C and harvested at stationary phase ($OD_{600}>1.2$). The collected cells were washed three times with sterile deionized water to remove completely nutrient. The cell pellets were then resuspended in sterile DI as a stock solution with a concentration of 10^9 CFU/mL ($OD_{600}=0.6$). The stock solution was then used to inoculate into the artificial secondary effluent water to working concentration of 10^6 CFU/mL. The numbers of *E. coli*, *E. faecalis*, *S. Typhimurium* in the feeding water solution and samples from the filtration column were enumerated using membrane filtration method. A serial dilution of samples was prepared by transferring 1.0 mL of sample into test tubes containing 9.0 mL of sterile 0.1x PBS buffer. 1.0 mL of the appropriate dilution was then filtered through 0.45 μ m GC-6 membranes. For *E. coli*, filtered membranes were placed onto mTEC agar plates and then incubated at 35°C for 2 hours and 44.5°C for overnight. For *E. faecalis*, filtered membranes were transferred onto into mEI agar plates followed by overnight incubation at 41°C. *S. Typhimurium* was determined by using Salmonella Shigella agar plates. Culture plates were incubated at 35°C for 24 hours.

4.2.2 Iron coated sand column setup

The column used in this study was made of the acrylic tube with $D_i=6.35$ cm and $H_{total}=185$ cm. The sand was dry packing intermittent 5.0 cm up to $H_{sand}=120$ cm. Gravel (3-4 mm) was used as support layer with a thickness of 5.0 cm. The empty bed volume is 3.8 L ($H=120$ cm). The pore volume of filter media was calculated from the water displacement in a graduated cylinder by subtracting the water displacement volume from packed bulk filter material volume, 1.52 L (porous ratio~0.4). Prior to start, the column

was slowly and intermittently saturated with DI water from the bottom prior to removing air phase trapped in the media.

Quartz sand was sieved to the size range of 0.6 to 1.1 mm. Sand was washed several times to remove the impurity on the sand surface. Additionally, the sand was soaked into concentrated 10 M HCl for 24 hours and rinsed 0.01 M NaOH to remove metal oxide and organic carbon. The sand was then rinsed with DI water until effluent pH unchanged ($\text{pH} \approx 8.0$) [217]. The clean sand was then coated using the procedure described by Mills et al. [140]. Briefly, 200g cleaned sand was added into a plastic bottle containing 20 g $\text{FeCl}_3 \cdot \text{H}_2\text{O}$ dissolved in 400 mL distilled water. A 30 mL of NaOH (0.5 M) was added into and followed by sequential addition of 1 mL NaOH (0.5 M) until pH reach 4.5-5.0. The mixture was shaken for 36 hours to allow completely coating onto sand surfaces. Iron coated sand was then rinsed with DI water and dry at 90°C for three times, and then saved in a clean container for later use.

The artificial secondary effluent was used to feed into the column. The composition of feeding water contained NaHCO_3 (96 mg/L), NaCl (7 mg/L), urea (6 mg/L), $\text{Mg}(\text{Cl}_2)$ (60 mg/L), KCl (4 mg/L), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (4 mg/L), peptone (32 mg/L), (2 mg/L), meat extract (22 mg/L) [218]. Sodium acetate was used as the only organic substrate with measured concentration 30 mg/L as TOC. The wastewater was applied to the top of the column using a peristaltic pump at the loading rate $95 \text{ L/m}^2\text{day}$, and the water flow was caused by gravitational force with a hydraulic retention time of 5 days.

Seed of anaerobic bacteria inoculated into the filtration column was taken from anaerobic enrichment for Waipahu soil sediment. A 100mL of freshwater medium

containing 2.5g/L NaHCO₃, 0.1g/L NaCl, 0.1g/L NaCl, 0.1g/L KCl, 0.1g/L MgCl₂.6H₂O, 0.1g/L CaCl₂.2H₂O, 1.5g/L NH₄Cl, 0.6g/L NaH₂PO₄, 0.1g/L MgSO₄.7H₂O, 0.005g/L MnCl₂.4H₂O, 0.001g/L NaMoO₄, and 0.05g/L yeast extract, 2.7g/L (10mM) sodium acetate were used to enrich anaerobic microorganisms [67]. For enrichment of iron reducing bacteria, 20 g of iron coated sand and 10 mM MgNO₃ were amended into serum bottles as electron acceptors for iron reducers and denitrifiers while HCO₃⁻, SO₄⁻ were removed out of freshwater medium. The medium was modified for sulfate reduction by increasing concentration of MgSO₄ to 10mM while it was removed from the medium for methanogens. 20 g of clean quartz sand was added to sulfate, nitrate reduction, and methanogenesis. 1.0 g of Waipahu soil was used as inoculum. The serum bottles were capped with rubber stopper and sealed with aluminum crimp. The anaerobic condition was established by flushing N₂ gas for 15 minutes into the head space for three times. The enrichment was incubated at room temperature under dark condition. 5.0 mL of a mixture of enrichment culture was added to the column along the column via effluent ports.

Samples were collected at different depths in the sand column during the experiment. The column was interrupted at the end of experiment and core sand samples of filtration media were collected right above the sampling ports at different depths. Sand samples were washed by two-step procedure described by Boehm et al. [219]. Briefly, 10 g of sand samples were added to a 250 mL flask containing 60mL 0.1x PBS and vigorously shaking by hand for 2 minutes and followed by 30s settling before decant the supernatant. Another 40 mL of PBS was added to sand and swirled for 10s followed by 30s settling. The supernatant was extracted for further analysis. All samples are processed

immediately after collection. An average concentration of three samples at each depth was used to present the adsorption of bacteria to iron coated sand slow sand filtration.

4.2.3 Biofilm development of iron reducer and adsorption experiment

Iron reducing bacterial biofilm experiment was conducted in Coy anaerobic chamber. Polystyrene slides (0.8x2.0 cm) were coated with ferric iron as following procedure. 5 M NaOH was added to 0.5 M ferric chloride (hexahydrate) until pH reached 4.5-5.0. The slurry was then dried at 110°C in the oven for 24 hours. The dried ferric oxide was ground into small particle and sieved through mesh N°60. The ferric oxide powder was then applied onto the plastic surfaces by using a super glue to support the binding. A thin layer of ferric oxide formed on the slide surfaces. The ferric iron coated glass slides were rinsed with DI before use.

IRB biofilm growth experiment was set up in Coy anaerobic chamber. The ferric iron coated polystyrene slides were placed in a 1.5 mL plastic tube and subsequently into a serum bottle. The enriched freshwater described above was added to the bottle and then capped with rubber, and sealed by the aluminum crimp. The air was removed and nitrogen was flushed into the head space to establish the anaerobic condition. A 1.0 mL iron enrichment culture was added into reactors. The reactor was incubated for 8 weeks, and the microbial iron reduction was verified by measuring the ferrous iron accumulation overtime.

E. coli BL21 carrying GFP gene was used model microorganism in the adsorption experiment. A single colony was grown in LB with 50 mg/L ampicillin for 18 hours. When bacteria growth reach exponential phase ($OD_{600} = 0.5$), inducer IPTG (200 μ M)

was added to induce the protein. Bacterial cells were then harvested and washed three times with sterile DI water and resuspended 0.1 M NaCl/ 0.05 M HEPES (pH 7.4) to obtain the concentration 2.8×10^6 CFU/mL. Adsorption of *E. coli* onto biofilm of iron reducers and ferric iron oxide surfaces was conducted in test tubes containing desired concentration of bacteria. The test tubes were incubated at room temperature for 40 minutes [220]. Attachment of *E. coli* with GFP gene was then visualized under Confocal Laser Scanning Microscopy (CLSM). Images were then analyzed using open source Image J 1.48 software.

4.2.4 Analysis

a. Ferrous iron

Fe^{2+} was measured using the colorimetric ferrozine assay following the procedure described by Lovley *et al.* [65]. Briefly, liquid samples (0.1 mL) were transferred into 5 ml of ferrozine (1 g/liter) in 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (pH 7). The mixtures were vortexed for 15 s and then filtered through a Nuclepore filter (0.2 μm). Filtrates were measured for light absorbance at wavelength 562 nm using a spectrometer (Hach DR/4000U). Fe^{2+} concentration was calculated based on standard curves established by using ferrous ethylene-diammonium sulfate as standard solutions.

b. TOC

Total organic carbon concentration in the samples was determined by using a TOC analyzer (Shimadzu, TOC V_{CSH}) with a Total Nitrogen detector. Organic carbon in water samples was converted into carbon dioxide (CO_2) by combustion tube filled with an oxidation catalyst and heated up to 680°C. Once the sample combustion product had

been cooled down and removed chlorine and other halogens, the CO₂ in sample combustion gas was determined by a non-dispersive infrared analyzer. A TOC-Control V software was used to analyze the analog detection signal to obtain the TOC and TN concentration.

c. Iron oxides

Iron oxide was determined used Fourier transform infrared spectrophotometry (FTIR) method describe by [221]. Liquid samples were collected in aeration and extract solid phase by centrifugation at 15000 g for 5 minutes. The solid phase was then dried at 103°C for 48 hours. The samples were then ground to powder subjected to FTIR analysis. Approximately 1 % in weight of samples was mixed with 0.2 g KBr powder and compressed into pellets using a hydraulic press. The equipment was used to analyze iron oxides. FTIR spectra were collect for 32 scans with 2 cm⁻¹ resolutions.

d. Enzymatic activities

Dehydrogenase assay (DHA) and fluorescein diacetate hydrolytic activities (FDA) were performed based on protocol described by Schnurer and Rosswall (1982) and Mermillod et al. (2005) [78, 222] to analyze the enzymatic activities within the sand filter. 9.0 g of collected wet sand was mixed well with 0.2 g CaCO₃ in 100 mL flasks. The mixture was amended by 3 ml of 1.0 % aqueous solution of 2,3,5-triphenyltetrazolium and then incubated at 37°C for 24 hours. The product, triphenylformazan (TPF) resulted from enzymatic activities was then extracted by adding 10 mL ethanol and shaking for 1.0 minute. The supernatant was filtered through a 0.45 µm membrane, and the filtrates were measured for light absorbance using spectrophotometer (Hach DR/4000U) at a wavelength of 485 nm. The enzymatic activity

was determined based on a standard curve established by using standard solutions of TPF. For FDA, 2.0 g of collected sand was used to mix with 45 mL of 60 mM phosphate buffer (pH 7.6) in a 100 ml flask. 100 μ L of FDA solution containing 10 mg/mL in acetone was added into the cultures. The mixture was then incubated for 1 hour on a shaker. The supernatant was extracted by centrifugation at 6000 rpm for 5 minutes and then measured the light absorbance using a spectrophotometer at a wavelength of 490 nm. The product of enzymatic conversion (fluorescein) was calculated based a standard curve established by using standard solutions of fluorescein.

4.3 Results

4.3.1 Reactor startup

The iron coated sand filter was operated for 60 days to establish the anaerobic condition. During the first 30 days, the hydraulic retention time (HRT) of the filter was 1.5 days. DO was measured for every two days during the first startup period (Figure A.6). The anoxic condition was quickly established in the column. However, the DO concentration was still remaining above 1.0 mg/L. For the next 30 days, the hydraulic was increased to 5.0 days, and resazurin was used as a redox indicator. During this second startup period, the effluent water was colorless at the bottom of the filter (Figure A.9), indicating that the anaerobic condition had been fully established inside the filter.

4.3.2 TOC removal

After the reactor startup, organic removal efficiency of the anaerobic/aerobic two stage bio-filters was determined by running the reactor for 120 days and quantify TOC concentration in the 1st stage influent, 1st stage effluent, and 2nd stage effluent (Figure

4.1A). The TOC concentration in the influent into the 1st stage anaerobic biofilter averaged at 37.8 mg/L (range: 26.8 - 48.8 mg/L), and the variability was likely caused during the preparation of influent water. The TOC concentration in the effluent of the 1st stage anaerobic biofilter averaged at 3.6 mg/L (range: 2.0-7.2 mg/L, standard deviation 1.6 mg/L), and the TOC concentration in the effluent of the 2nd stage aerobic biofilter averaged at 2.2 mg/L (range: 1.5-4.7 mg/L, standard deviation:0.8 mg/L). High removal efficiency of TOC was achieved in the 1st stage anaerobic biofilter ($89.43 \pm 6.4 \%$) (Figure 4.1B). The additional removal in the 2nd stage aerobic filter further enhanced the TOC removal, resulting in cumulative removal percentage of $93.6 \pm 3.5 \%$.

4.3.3 Removal of microbial contaminants

The removal of microbial contaminants by the 1st stage anaerobic biofilter was determined by spiking laboratory-cultivated *E. coli*, *E. faecalis*, and *S. Typhimurium* cells in the influent and monitoring bacterial concentrations along the filter (D1, D2, and D3) and in the effluent. The mean concentrations of *E. coli*, *E. faecalis*, and *S. Typhimurium* cells in the influent (C_I) were 2.2×10^6 , 1.0×10^6 , and 2.1×10^6 CFU/mL, respectively, over the 50 days experimental course, which equals to 10 pore volumes. The anaerobic biofilter resulted in the considerable removal of all three bacteria tested and exhibited higher removal of *E. faecalis* than of *E. coli* and *S. Typhimurium* (Figure 4.2). The average log reduction of *E. faecalis* cells was -6.0 ± 0.4 , and there was no clear breakthrough over the experimental course. For *S. Typhimurium* and *E. coli*, a breakthrough occurred at 1.5 pore volume, and then subsequently fluctuated significantly. In spite of the fluctuation, significant removal of *E. coli* ($\log C_E/C_I: -4.4 \pm 1.0$) and *S. Typhimurium* ($\log C_E/C_I: -2.9 \pm 0.6$) were still observed.

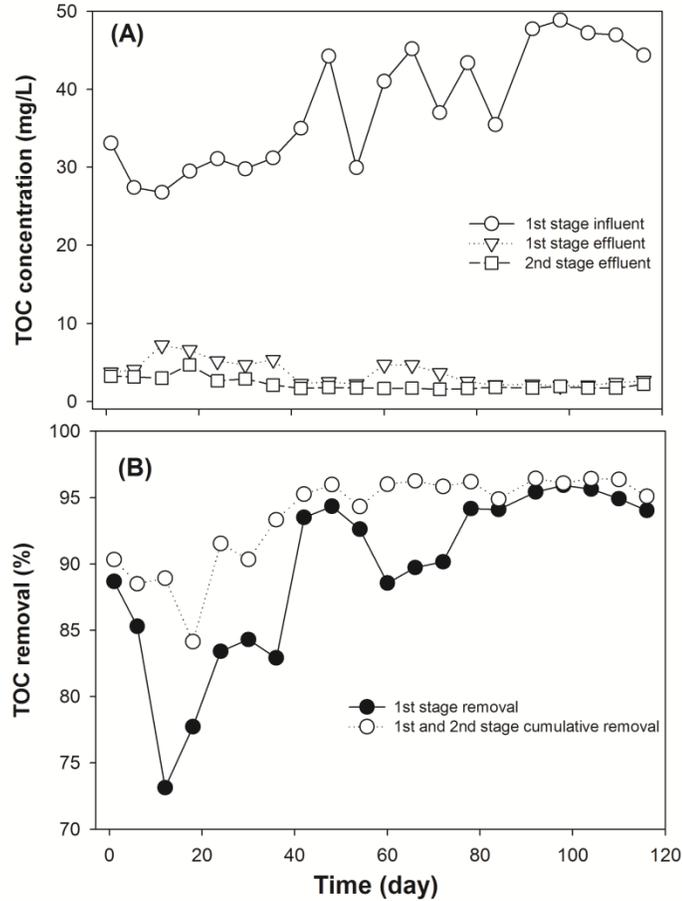


Figure 4.1. TOC concentrations (A) and removal (B) in the two-stages filter column. 1st filtration stage: anaerobic iron coated sand filter, 2nd filtration stage: aerobic uncoated sand filter.

Water samples were also collected from the different depths of the 1st stage anaerobic filter to determine bacterial concentrations (Figure A.8). The data were used to calculate the average removal efficiency of bacteria at different depths (Figure 4.3). Bacterial removal was observed throughout the filter depth, and large percentages of bacterial removal were observed at the first layer of the anaerobic filter.

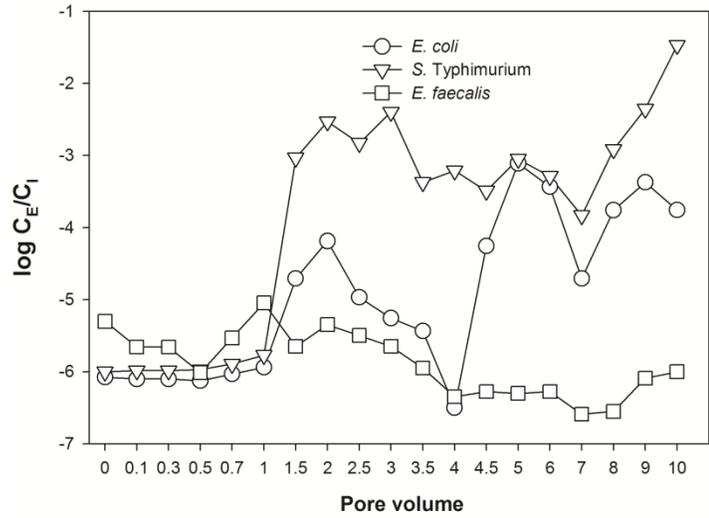


Figure 4.2. Comparison of bacterial concentration in the influent (C_I) and the effluent (C_E) of the 1st stage anaerobic biofilter.

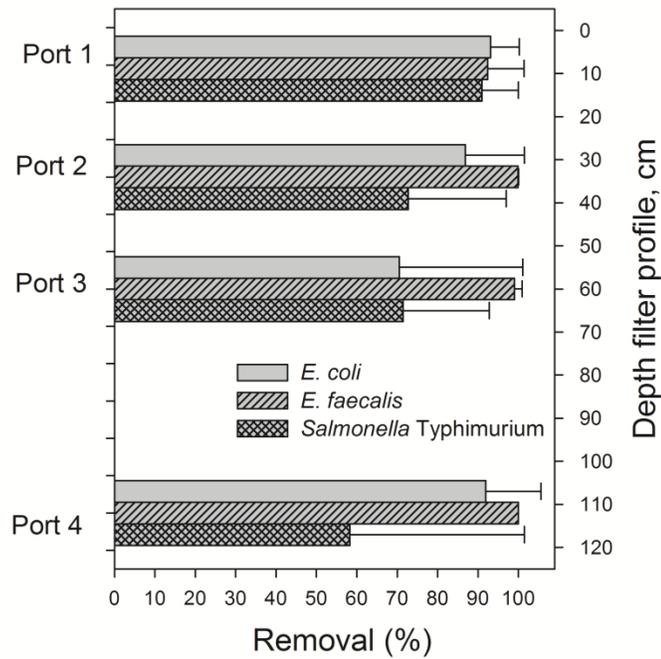


Figure 4.3. Removal efficiency at different depths of the anaerobic filter

Table 4.1. Cumulative removal efficiencies of microorganisms along the filtration depth. D1: biolayer + zero valent iron layer; D2: 30cm; D3:60cm; Effluent:120cm

Depth	Removal efficiency (%)					
	<i>E. coli</i>		<i>E. faecalis</i>		<i>S. Typhimurium</i>	
	Average	STD	Average	STD	Average	STD
D1	92.9	8.6	93.1	4.0	91.2	7.3
D2	99.6	0.5	100.0	0.0	98.7	0.5
D3	99.8	0.2	100.0	0.0	99.7	0.2
Effluent	100.0	0.0	100.0	0.0	99.9	0.1

4.3.4 Overall microbial cell mass balance

At the end of the experiment, the sand filter was disassembled to collect sand samples, and bacterial cells retained in the sand samples were enumerated. Figure 4.4 showed that more bacteria cells were retained in top portion of the biofilter than in lower portions. This result revealed that iron coated sand was effective to absorb bacteria. Each bacterial species was also found at different levels on the sand surfaces throughout the whole filtration depth. Specifically, *E. faecalis* was the species that has the lowest number while *S. Typhimurium* was the species that had the highest number in the sand columns.

Although bacteria concentration in the feeding solution for three species was approximately similar, the concentrations of each species absorbing to a solid phase and concentrations in aqueous solution were different. *E. faecalis* was detected in sand media, but there is mostly no detection in water, suggesting that *E. faecalis* was strongly adsorbed to iron coated sand surfaces. The results of mass balances showed that large

numbers of trapped *E. faecalis* was inactivated in the column (Table 2). *E. coli* was also strongly absorbed onto the ferric oxide coated sand surfaces. The concentration in aqueous solution at lower depth was 1.6 log differences less than that adsorbed onto media surfaces while there was an insignificant difference in the upper portion. *S. Typhimurium* was predominant in iron coated sand media. A low reduction rate of *S. Typhimurium* was achieved in comparison to *E. coli* and *E. faecalis*. However, the overall removal efficiency for the entire testing time was 99.9 %.

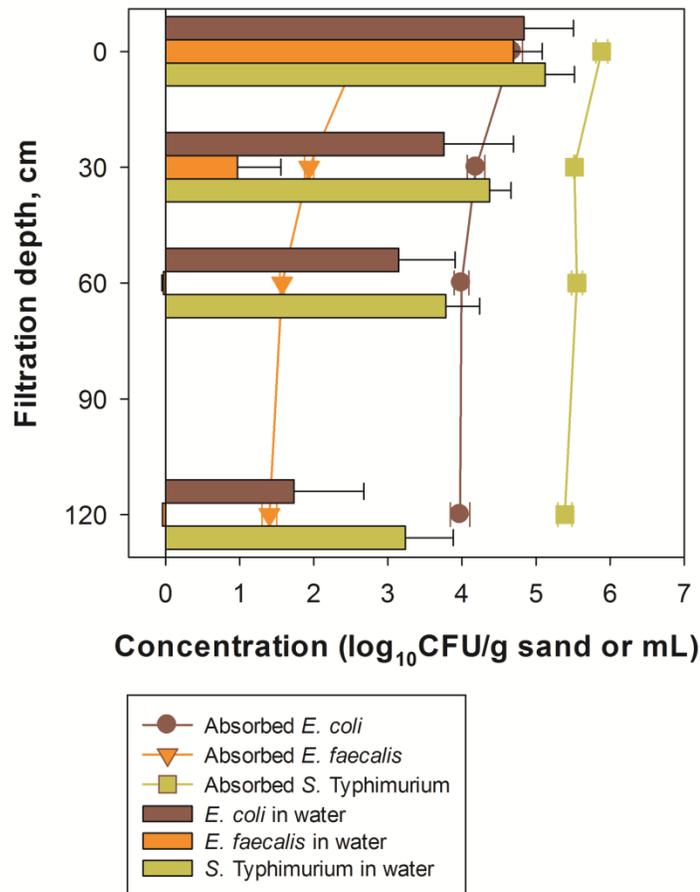


Figure 4.4. Comparison total absorbed bacteria on the iron coated sand and total bacterial concentration in water samples.

4.3.5 Microbial activities in iron coated sand column

Activities of microbial communities in the 1st stage anaerobic biofilter were quantified by hydrolytic activities and dehydrogenase analyzed from extracted sand samples collected at the end of the experiment. Figure 4.5 shows that the average TPF and fluorescein on the top surfaces and at different filtration depths. The TPF and fluorescein concentrations were 20.3 $\mu\text{g/g}$ sand and 6.3 $\mu\text{g/g}$ sand per hour in the top surfaces, which indicated that highest activities of microbial communities were obtained in the top portion of the filter. At lower filtration depth, the TPF and fluorescein concentration were decreased dramatically, suggesting that the microbial activities were decreased to the bottom of filter column. Hydrolytic activities maintained higher than dehydrogenases in the lower zone of filtration media.

Table 4.2. Mass balance of bacteria cells in the 1st stage anaerobic biofilter over the experimental course

	Total bacterial cells (CFU)		
	<i>E. coli</i>	<i>E. faecalis</i>	<i>S. Typhimurim</i>
Influent water	4.35E+10	2.28E+10	4.28E+10
Effluent water	4.56E+06	ND*	8.83E+07
Live cells retained on sand	6.89E+07	4.11E+05	1.68E+09
Inactivated cells	4.34E+10	2.28E+10	4.10E+10
Inactivated percentage (%)	99.8	100.0	95.9

* Not Detected

4.3.6 Aerobic filtration stage

There was no detection of ferrous ion in the second stage filtration due to oxidation of ferrous ion to form ferric oxide under aeration condition. The oxidation of

ferrous ion in the effluent of the anaerobic filter to ferric oxide was showed as the formation of reddish color suspended solids. This solid phase was precipitated in the aeration tank and retain sand media while passing through sand media. The formation of ferric oxide in aerobic filtration stage was analyzed using FTIR method.

High reduction of *S. Typhimurium* and *E. coli* was continuously achieved in the second stage filtration (Figure 4.6). Specifically, 3.0 log units removal of *S. Typhimurium* was obtained. The highest concentration of *S. Typhimurium* detected in the 2nd stage effluent was 400 CFU/100mL. There was mostly no detection of *E. coli* in the effluent of the second filtration stage.

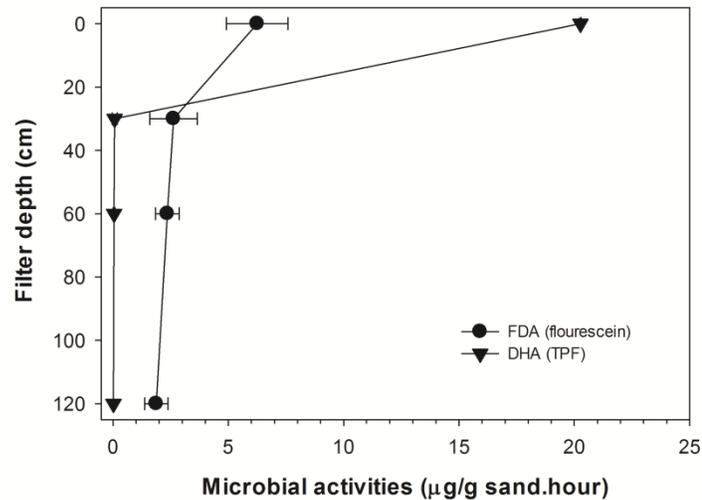


Figure 4.5. FDA and DHA enzymatic activities at different depths of the anaerobic column.

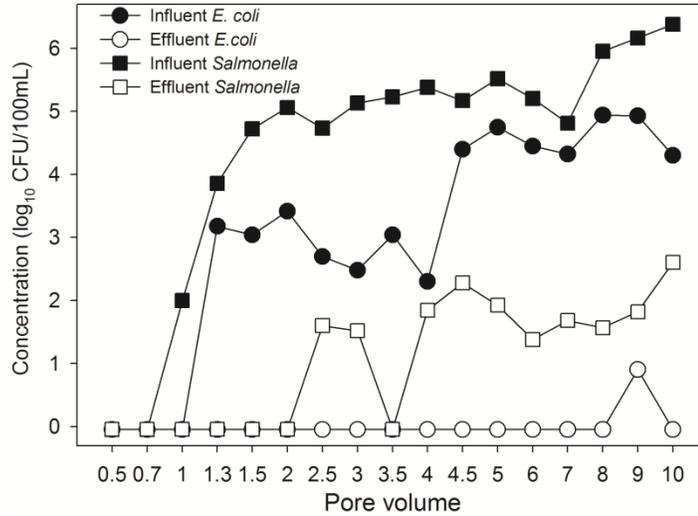


Figure 4.6. Breakthrough curves of *E. coli* and *S. Typhimurium* in aerobic filtration.

4.4 Discussions

4.4.1 Organic substrate removal

Removal of dissolved organic carbons (DOCs) is limited in old fashion SSF. It was reported that reduction of DOCs in SSF was obtained at the top portion of filtration media and decreased at lower depth filtration [78]. Removal of organic carbon in SSF was due to activities of heterotrophic bacteria colonized in the top portion of the filtration bed. Weber-Shrink and Dick suggested that physical-chemical and biological ripening that result in the removal of particles would not be obtained if raw water containing a low concentration of DOC and bacteria [30].

Numerous studies showed that varieties of organic compounds were degraded during the MIR in the field and laboratory [73, 75, 90, 91, 223, 224]. Our results showed that removal of acetate achieved 90 % in artificial secondary effluent water. The results

indicated that reduction of TOC in iron coated SSF was facilitated by activities of microbial communities in the column. It is interesting that the removal rate of TOC in our study was much higher than old fashion SSF [207]. The results suggested that the development of MIR under anoxic or anaerobic at lower depth filter enhance the oxidization of DOCs.

4.4.2 Reduction of bacteria in iron coated filter

Bacterial removal in SSF systems has been confirmed in numerous studies [25, 30, 163, 225, 226]. The removal efficiencies depend on several factors such as specific surface areas of sand grains [25, 225], hydraulic loading [27], properties of feeding water [30, 227, 228]. The highest removal efficiencies of fecal bacteria in reclaimed water by a SSF were reported up to 1.9-2.6 log units for *E. coli* and 1.9-3-log units for *E. faecalis* [25]. Our observation on bacterial reduction in anaerobic-aerobic two-stage filter was significantly higher than data obtained in a previous study using biosand filters [25]. The possible explanation was due to higher filtration depth used in our study in comparison to 50 cm used by Langenbach et al. (2009) [25]. The iron coated sand had been showed to absorb more bacteria than the uncoated surfaces [39, 40]. Lukasik et al. (1999) reported that the ferric and aluminum oxide coated sand removed 99.999 % of *E. coli* [39], which is comparable to our study (Figure 4.2). The results showed that there were different removal rates for each bacterial species (*E. faecalis* > *E. coli* > *S. Typhimurium*). Our experimental results suggested that these tested bacteria might have different survival rate in the iron reducing environment.

Adsorption and inactivation might be attributable to the removal of bacteria in iron coated sand filter operated under iron-reducing condition. The electrostatic interaction was proposed to be the primary mechanism for the reduction of microorganisms in iron based sand filter.[38, 140]. Our data showed that *E. coli* quickly adsorb to the ferric oxide surfaces and biotic surfaces of MIR (Figure 4.7 and Figure A.7). A different form of iron oxide resulted in different interaction. It was interesting that the biotic surface of MIR also appeared to have a high adsorption affinity to *E. coli*. The cell-cell interaction could be the case of bacterial attachment. It was revealed that the threshold adsorption of bacteria to iron coated sand reach approximately 7×10^8 cells/gram sand [140]. Bacterial attachment of bacteria to iron coated surface was possibly stronger and irreversible due to bonding between bacterial cells and iron oxides [229] via C-metal or O-metal bonds. A previous study revealed that bacteria bound ferric iron could lead to the loss of cellular membrane integrity of attached bacteria [52]. The presence of ferrous ion in either aerobic or anaerobic condition resulted in inactivation of the bacteria [113]. Thus, our filtration system may attributable to inactivation of bacteria by ferrous products during the filtration process operated under the iron reducing condition.

4.4.3 Microbial activities

The microbial activities facilitated the generation of the ferrous ion and development of biomass within the filter media by using the acetate as the electron donor and Fe^{3+} as the electron acceptor in the anaerobic iron coated sand filter. The removal of TOC was increased and reached steady state after 3 months operated under the iron reducing condition (Figure 4.1B), suggesting that microbial activities in the filter column

has fully established in the filter. A previous study revealed that the anaerobic iron reduction in slow sand filtration amended with bulk liquid ferric oxide showed high activities of microbial iron reduction coupled with the oxidation of organic substrates [230]. The Fe^{2+} product in our system was small and not comparable to the data attained in the previous study [230] because different configuration and form ferric iron were used in this study. According to Ivanov (2010), various forms of ferric iron forms resulted in varieties of reduction rates under the same condition [231].

Our study showed that the protein contents were attained more at the top portion of filtration media. Dehydrogenase was highly observed in the upper filtration zone, but it drastically decreased with the depth. However, activities of FDA hydrolysis slightly decreased with respect to filtration depth. It was report that the enzymatic activities strongly correlated to organic degradation, oxygen consumption, and nitrate reduction [78]. This study addressed that the microbial activity was highly achieved at the oxic zone of the top area of porous media, whereas it sharply decreased at the lower depth where the anaerobic condition was established. However, the biomass estimated by protein contents varied among operation conditions [36]. Mermillod-Blondin et al.(2005) reported that the protein contents in the porous media appeared to be highest the top and bottom of the filter, but they remained small concentration in between. Since protein content is complex microbial variable including death and alive cells, different bacterial species, microbial substances, our results showed that the DHA and FAD could response to various activities of bacterial species along the filter depth.

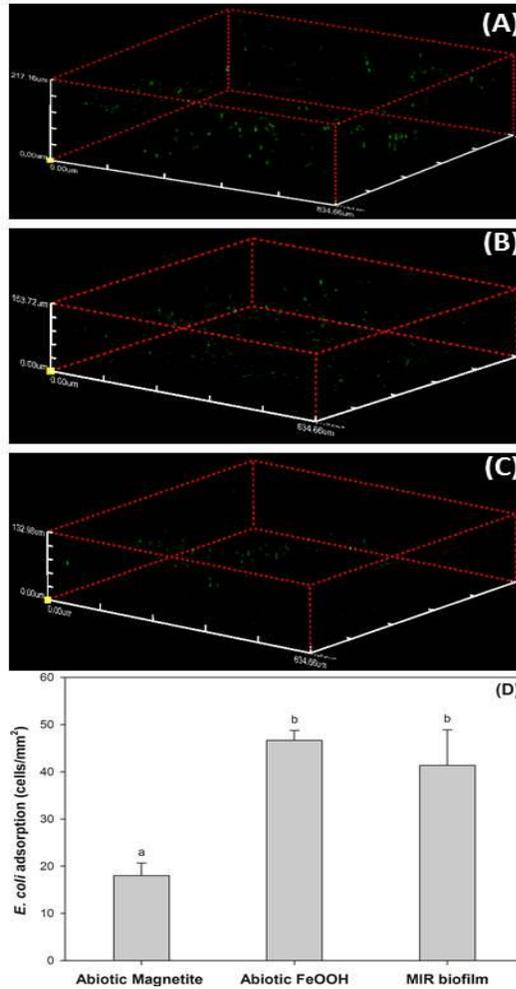


Figure 4.7. Adsorbed *E. coli* cells onto different abiotic surfaces of iron oxides and the biotic surface of MIR.

4.4.4 Iron phase

Ferrous production in the effluent of anaerobic filter potentially served as in-situ flocculation and sedimentation when it is oxidized to form ferric oxides. The oxidation of ferrous ion by dissolved oxygen in the water was confirmed in previous studies [232, 233]. Our data showed that the remaining numbers of *E. coli* and *S. Typhimurium* in the anaerobic filtration was highly removed in the aerobic filtration. Association of bacterial cells to iron-containing flocs followed by sand filtration led to the efficient removal of

microorganisms in the second stage filtration. A previous study revealed that the formation of ferric iron-containing flocs was able to entrain bacterial cells [211]. Another study also demonstrated that ferric oxides exhibit high adsorption affinity for bacterial cells [45]. This interaction possibly explains the high removal of microorganism in the second stage filtration in which ferrous ion was oxidized to form iron-containing flocs. It was demonstrated that lepidocrocite (γ -FeOOH) was formed during oxygenation of ferrous ion in neutral condition [233]. Analysis of precipitation of particles retained on sand and aeration compartment in the second stage filtration showed that various forms of iron oxides were detected (Figure A.12). The ferric oxide compounds were detected in the wide range of wavelengths. This may indicate that more than one compound of ferric oxides was formed during oxygenation process. In comparison to FTIR spectra of ferric oxides, different compounds of iron oxides were proposed to form. In addition, Figure A.12 shows that the absorbance of cellular materials such as carboxyl and proteins was also detected in the iron-containing flocs. The range of light absorbance was similar to that in a previous study at the wavelength of 1300-1750 cm^{-1} [229]. The result of in this analysis confirmed that bacteria were attracted and associated with the formation of iron oxide particles during the aeration process.

4.5 Conclusions

The anaerobic-aerobic two stage filter appeared to be a strong candidate to remove microbial contaminants in reclaimed water. The results attained in this study revealed that the coated sand filter was very efficient to remove *E. coli* and *E. faecalis* and *S. Typhimurium*. The ferric iron that covering the sand surfaces not only increased the bacterial attachment, but it also served as an electron acceptor under anaerobic

condition. Our result showed that ferrous iron was produced during the process. This metabolic product might attribute to bacterial removal. The integrated both anaerobic and aerobic filtration provided an improvement of system performance converting iron from a soluble form to slurry which further facilitated the removal of bacteria by adsorption, sedimentation, and filtration.

CHAPTER 5. REMOVAL OF ESTROGENS, ANTIBIOTICS, AND PHARMACEUTICALS IN AN ANAEROBIC IRON COATED FILTER

Abstract

Most effluents of wastewater treatment plants contain contaminants of emerging concerns (CECs). Inadequate treatment of these chemicals in reclaimed water may create long-term problems for the environment. Microbial iron reduction (MIR) has been revealed as a robust process that couples with the oxidation varieties of organic compounds. The objective of this study aimed to examine the capability of anaerobic iron coated sand filter in removing of CECs. We assume that the CECs will adsorb to the biomass and be oxidized in MIR process where iron oxides served as the electron acceptor. The results showed that the removal efficiency of CECs passing through iron coated sand column varied one compound to another. Antibiotics were completely removed from the aqueous solution. Estrone (E1) and 17 β -estradiol (E2) were highly removed, but estriol (E3) appears to have a little reduction during the experiment. The removal of pharmaceuticals depended on chemical compounds. There was a high reduction of caffeine, but little removal of carbamazepine (CBZ) and gemfibrozil (GFZ) was obtained in the anaerobic filter column. Our results demonstrated that anaerobic condition naturally established in filter column provides favor reducing environment for MIR. Different chemical compounds of CECs showed different removal during the treatment process. The anaerobic iron coated sand could be a potential means for tertiary treatment to improve the quality of reclaimed water.

5.1 Introduction

Contaminants of emerging concerns have recently attracted much attention of research interests in the state and other regions. The detected concentrations of these chemicals in water bodies and sediments are in trace levels, but they appear to have a potential risk to aquatic life. Exposure of a freshwater fish to nanogram level of concentration of estrogenic compounds causes the synthesis of vitellogenin in male fish [234, 235]. Moreover, the presence of CECs in water may lead bacteria adapting antibiotic resistant that pose the greatest potential risk to public health [9]. Several CECs are refractory organic compounds. These contaminants exist for a long time and deposit to the sediment when they enter the water environment.

Wastewater is the primary source CECs that detected in the surface water and the sedimentary environment. Alongside the disinfection byproduct, much attention was raised for the safety use of treated wastewater containing CECs [13]. Major concerned CECs are often referred to personal care products, pharmaceuticals, hormone steroids, and antibiotics. These micropollutants were detected in the primary effluents from domestic wastewater treatment plants (WWTPs) [235, 236]. CECs were also found in surface water systems that receive discharged sewage and treated wastewater [237]. The removal of CECs from aqueous solution using conventional wastewater treatment methods depends on the process and specific compounds. Membrane technology exhibited to have a high removal efficiency of CECs [14, 238]. Previous studies also showed that CECs were effectively removed in the advanced oxidation process [218, 239]. The conventional wastewater treatment processes also removed some CECs, but the removal rate substantially depended on the types of chemical compounds and biological

conditions [236, 240]. The inexpensive bio-filtration processes like soil and sand filter only removed biodegradable compounds in the laboratory-scale columns [241, 242]

Iron oxide coated sand filter has been recently investigated to remove microbial contaminants and heavy metals. Metallic oxides containing filtration media showed an advantage to enhance the bacterial colonization and activities in the filtration process [54, 210]. During SSF operation, the anaerobic condition was naturally established, and analogous to the sedimentary environment [78] where MIR occurred. Oxidation of aromatic compounds was addressed in an iron-reducing environment in sub-surfaces [213]. Numerous studies showed that varieties of organic compounds were degraded during the MIR in the field and laboratory [73, 75, 90, 91, 223, 224]. Dichloro diphenyl trichloroethane (DDT), estrogens, and toxic vegetable oil also showed to have removal in MIR environment [231, 243, 244].

Reclaimed water must be safe for irrigation and reuse. Treated wastewater should be free of CECs. Therefore, a new treatment approach is proposed to remove these trace contaminants from the aqueous solution. We hypothesized that biomass developing in anaerobic filter would absorb CECs that is then utilized as an electron donor in MIR. Our objective was to examine the removal of CECs in artificial secondary effluents through the anaerobic iron coated sand filter.

5.2 Materials and methods

5.2.1 Iron-coated sand preparation

Quartz sand was coated by amorphous ferric oxyhydroxide (FeOOH) following the procedure described by Mills et al., 1994 [140]. Sand was heated at 550°C for 3 hours

and then rinsed several times with DI water to remove organic matters. Trace metal was washed by soaking the sand in concentrated 10 M HCl for 24 hours, then rinsing in 0.01 M NaOH, and finally rinsed with DI water until the effluent pH reached 8.0 ± 0.1 . Sand was dried at 110°C , and then stored in a clean bottle for later use. The cleaned dry quartz sand was immersed in 400 mL of FeCl_3 solution (50 g/L of $\text{FeCl}_3 \cdot \text{H}_2\text{O}$, pH 1.9). A 30 mL of NaOH (0.5 M) was added instantaneously followed by gradual addition of 1 mL NaOH (0.5 M) until pH reaches 4.5-5.0. The mixture was then shaken for 36 hours to allow further coating of FeOOH onto sand surfaces. Iron coated sand was then rinsed with DI water, dried at 90°C , and saved in a clean bottle for later use.

5.2.2 Column configuration and operation

The column used in this study was made of the acrylic tube with $D_i = 6.35$ cm and $H_{\text{total}} = 185$ cm. The sand was dry packing intermittent 5 cm up to $H_{\text{sand}} = 120$ cm and gravel support layer was 5 cm (3-4 mm). The empty bed volume was 3.8 L. The pore volume of filter media was calculated from the water displacement in a graduated cylinder by subtracting the water displacement volume from packed bulk filter material volume of 1.52 L. Prior to starting the column was slowly and intermittently saturated with DI water from the bottom prior to removing air phase trapped in the media. Water flow direction is downflow mode. Hydraulic retention time was 5 days to maintain anaerobic condition.

5.2.3 Wastewater preparation

Artificial reclaimed water was used to the column and prepared based on composition described in previous study [218]. The solution contained NaHCO_3 (96

mg/L), NaCl (7 mg/L), urea (6 mg/L), Mg(Cl₂) (60 mg/L), KCl(4 mg/L), CaCl₂.2H₂O (4 mg/L), peptone (32 mg/L), (2 mg/L), meat extract (22 mg/L). Sodium acetate was used as a carbon source. In this study, three estrogenic compounds, three pharmaceuticals, and one antibiotic were selected to represent the CECs in the reclaimed water. All chemicals were purchased from Sigma-Aldrich. The stock solution was prepared by diluting each compound in methanol at the concentration 1.0 g/L. The sub-stock solution of CECs was prepared by mixing all CECs in one vial to the final concentration of 10 mg/L for each. The mixture was then added to the artificial effluent wastewater to the concentration of 50 µg/L for feeding water.

5.2.4 Column operation and water sampling

The artificial secondary effluent was applying to the filter at the top by using a peristaltic pump at the flow rate 0.2 mL/min. Water samples were collected from the sampling ports at different filter depth (P1, P2, P3, effluent). All samples were processed within 24 hours after collection.

5.2.5 Chemical analysis

Analysis of selected CECs were performed using an HPLC system (Thermo Finnigan, Waltham, MA) coupled with a photodiode array (PDA) detector (Shimadzu, Columbia, MD) having a detection limit of 0.1 µg/L. Acetonitrile and deionized (DI) water were used as the mobile phase.

5.3 Results

5.3.1 Estrogenic compounds

The breakthrough curves (BTCs) of the CECs were achieved from the ratio between the concentration in samples collected from the sampling ports over time and the influent concentration (50 µg/L). Figure 5.1 presents the BTCs of estrogenic compounds including E1, E2, and E3 at a different depth for the filter. The results showed that there was no BTC for E1 and E2 at all depth for the entire course of experiments. E1 and E2 were greatly removed from the influent. However, the concentration of E1 was still detected approximately 5 µg/L in the lower part of the filter depth. Detection of E1 in the lower zone could result from the biotransformation of E2 in the upper filtration media. Figure 1B showed that E2 was mostly undetected, suggesting that the biodegradation of E2 might be attributable to the appearance of E1 product. Unlike to E1 and E2, E3 was recalcitrant while passing through the columns. The BTC of E3 was quickly achieved at the initial operation time at the top filter and slowly occurred at a lower depth. The data indicated that E3 was unlikely reduced during the treatment process. The concentration of E3 slightly increased over time in the effluent, suggesting that E3 might be slowly degraded. Adsorption and partition of E3 to the biomass might occur in the filter media.

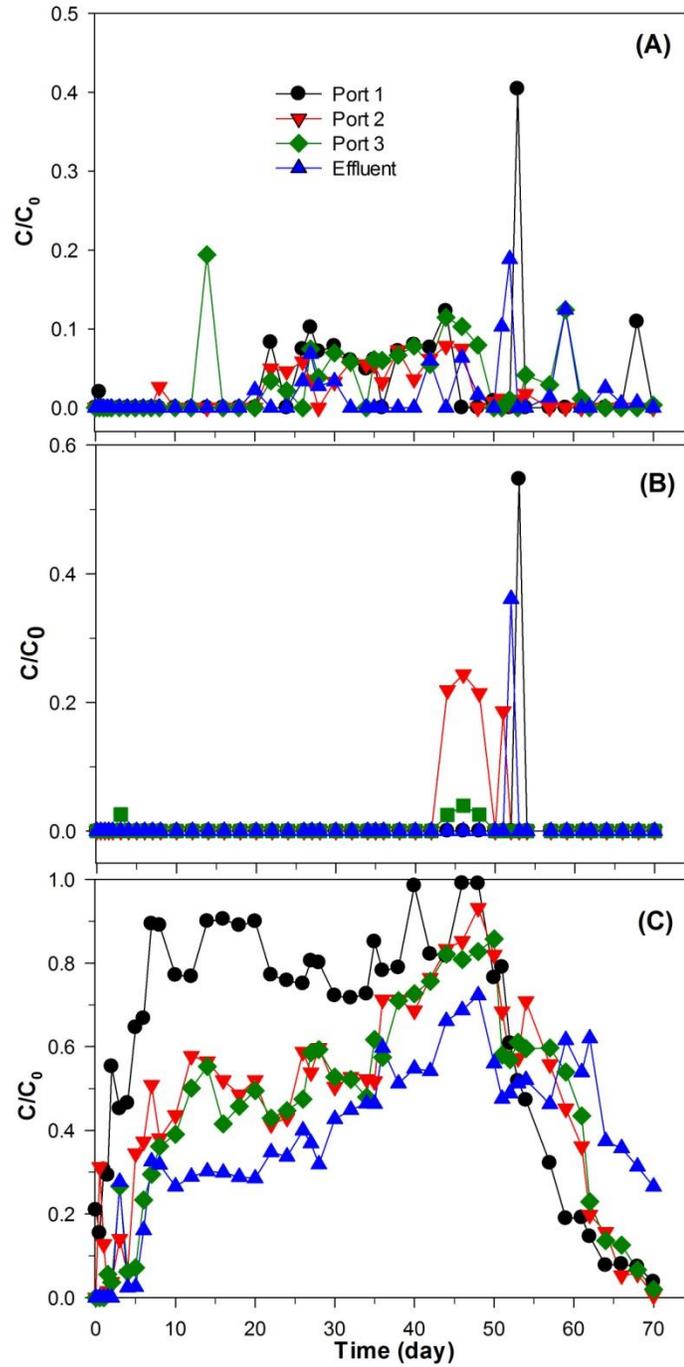


Figure 5.1. The BTCs of estrogenic compounds including Estrone (A), 17 β -Estradiol (B), and Estriol (C) with the influent concentration of 50 $\mu\text{g/L}$ at different filter depths.

5.3.2 Removal of pharmaceuticals

Figure 5.2 shows the BTCs of the selected pharmaceuticals at different layer depths of the filter. For caffeine, the BTC was quickly obtained for top filtration layer. The detected concentration of caffeine in the samples collected from P1 was mostly reached the input level after breakthrough. At the lower depth, the caffeine had an ease reach the breakthrough point. The behavior of caffeine at P1, P2 and P3 was likely similar to E3. The result suggested that caffeine was unlikely degraded in the upper filtration zone. However, there was no BTC of caffeine for the effluent port, indicating that caffeine was being removed while passing the lower filtration media. Since caffeine mostly appeared at the top sampling ports more rapidly than the lower ports, it might absorb to the biomass formed in the filter media at the bottom and slowly biodegraded. Our results showed that the concentration of caffeine in the effluent port ranged from 0-4.9 µg/L. The anaerobic microbial iron reduction might be responsible for the degradation of caffeine in the experiment.

The fate and transport of CBZ through the anaerobic iron coated sand filter were showed in Figure 2B. The concentration of CBZ exponentially increased in P1, and the C/C_0 ratio reached 0.8-0.9 at the steady state condition. The result showed that there was a little or no removal of CBZ at the top filtration layer. At lower sampling ports, CBZ partially appeared in the water samples collected from P2, P3, and effluent ports. The slowly increasing concentration of CBZ over time at the lower ports suggested that CBZ might absorb to the biomass and possibly minor degraded. At the end of the experiment, CBZ concentration in the effluent reached 80 % of the input level. The results showed

that carbamazepine was not effectively removed in an iron-reducing environment in a column.

Among the three selected pharmaceuticals, the BTCs of gemfibrozil at different depth were unpredictable. GFZ appeared to have a strange behavior in comparison to caffeine and CBZ. At the upper portion of the filter, GFZ concentration rapidly increased within five days and then slightly decreased over time. The BTCs at P3 and the effluent ports occurred after 20 days. GFZ concentration in the water samples exponentially increased, and the maximum level was higher than that in upper zone filtration. The strange occurrence of GFZ was possible due to the absorption and retention in the filtration media. The behavior of GFZ may indicate that this compound was bound to the biomass and then released rather than biodegradation.

5.3.3 Antibiotics

Antibiotics appeared to have similar behavior to E1 and E2. There was likely no BTC for triclosan. A small concentration of triclosan was detected in all samples at different depth. The result suggested that triclosan was completely degraded during the treatment process.

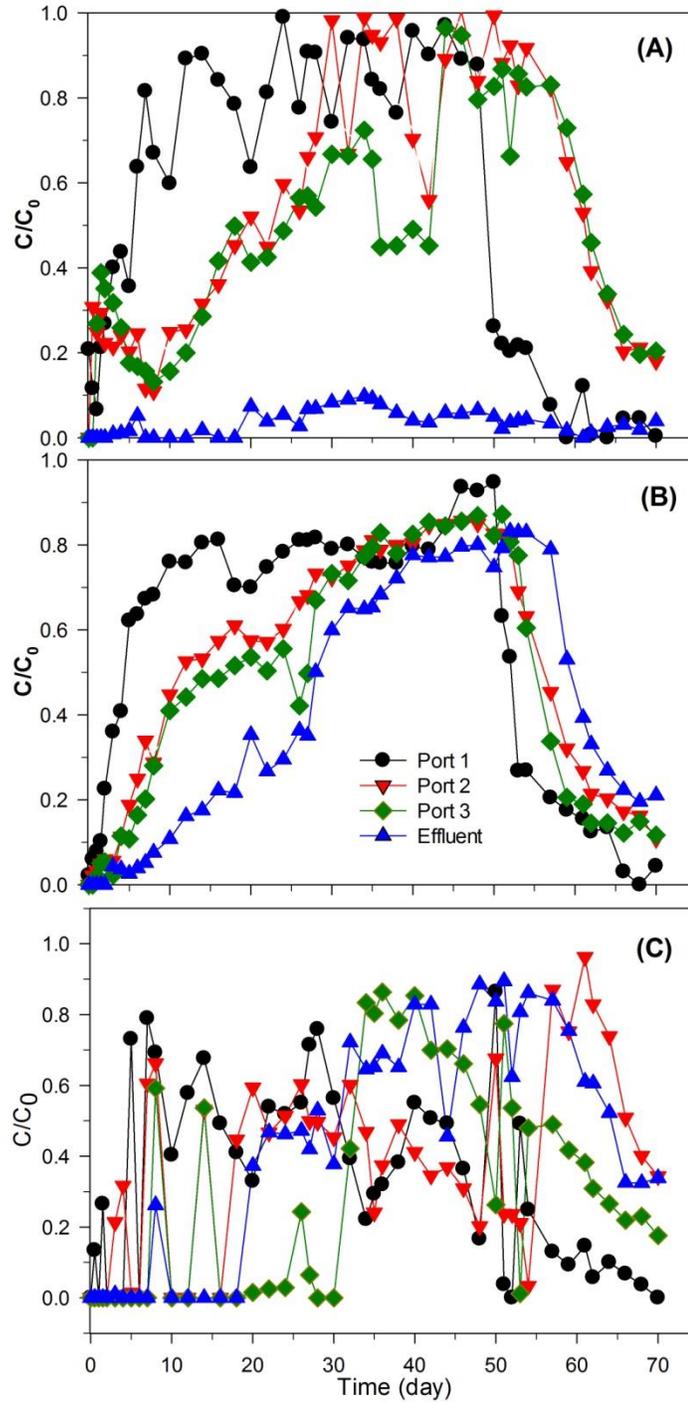


Figure 5.2. Breakthrough curves of caffeine (A), carbamazepine (B), and gemfibrozil (C) with the influent concentration of 50 $\mu\text{g/L}$ at different filter depths.

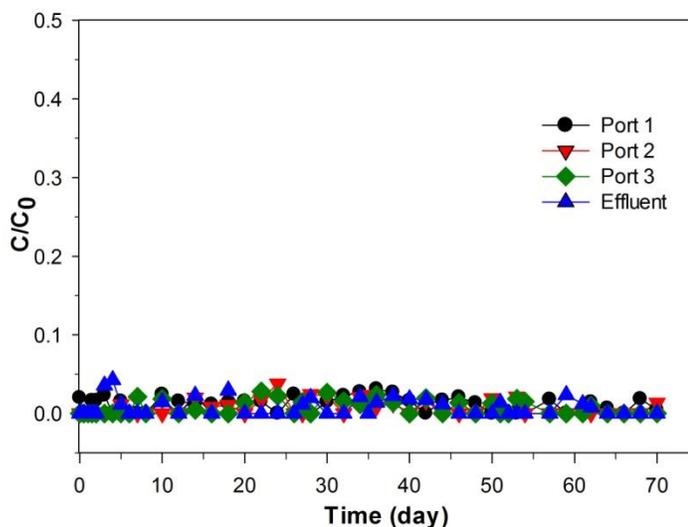


Figure 5.3. The BTC of triclosan with the influent concentration of 50 µg/L at various filter depths.

5.3.4 CECs' removal efficiencies in anaerobic FeOOH coated sand filter

Figure 4 showed the removal efficiencies of the estrogenic compounds, pharmaceuticals, and antibiotics in the FeOOH coated sand filter. The results showed that the removal of CECs varied among the selected compounds. For estrogens, E1 and E2 were highly removed from the water while the removal efficiency of E3 decreased over time (Figure 4A). The average removal efficiencies of E1 and E2 for the entire time course of the experiment were $98.3 \pm 3.8\%$ and $99.2 \pm 5.3\%$. The results indicated the filter column removed E3 at a low rate. The average removal efficiency of E3 was $63.4 \pm 20\%$ during the experiment. The high loading of E3 over time led to an increasing the concentration levels in the effluent water. For the pharmaceuticals, the filter was very efficient to remove caffeine from the feeding water. The average removal efficiency was $96.3 \pm 3.3\%$. The lower layer was attributable to the reduction. For CBZ and GFZ, the removal efficiencies were decreased over time during the treatment process. The similar trend of the removal efficiencies between CBZ and GFZ suggest that these contaminants

were resistant to degradation in the iron reducing environment. The FeOOH coated sand filter effectively removed the antibiotics from the feeding water. The average removal efficiencies of triclosan were 99.2 ± 1.1 %.

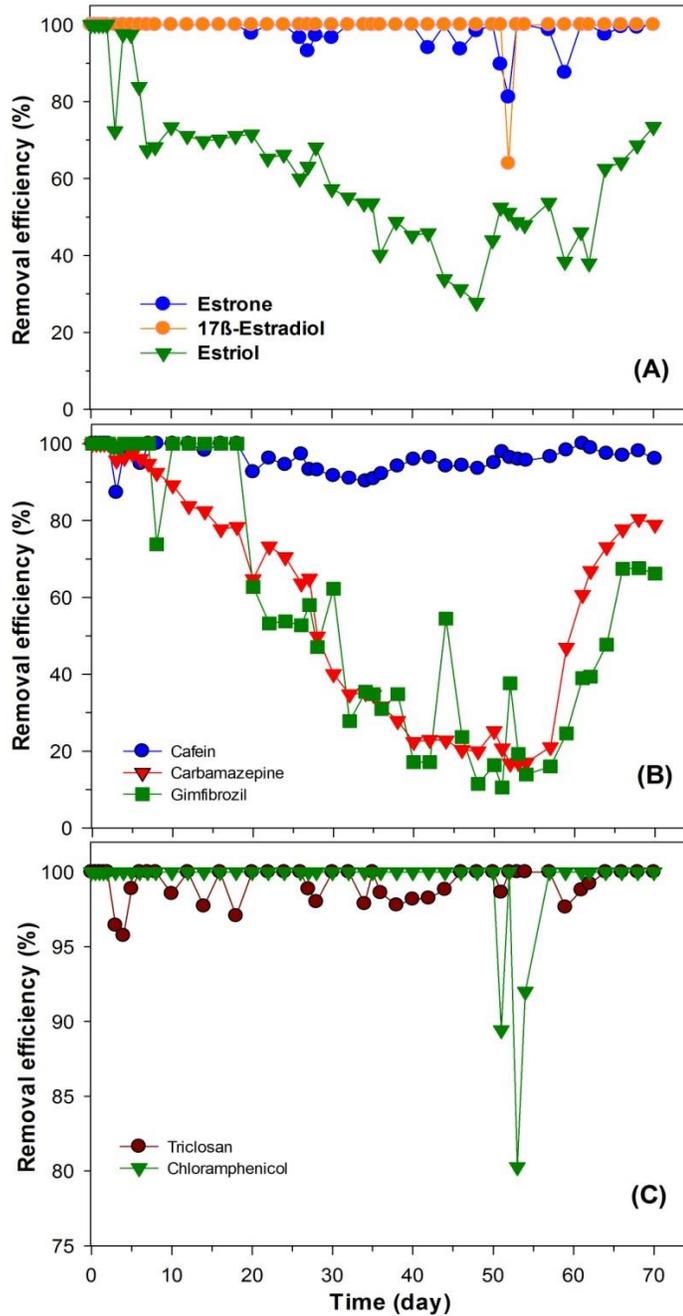


Figure 5.4. Removal efficiencies of estrogenic compounds (A), pharmaceuticals (B), and antibiotics (C) by the anaerobic FeOOH coated sand filter.

5.4 Discussions

5.4.1 Removal of estrogenic compounds

The low-cost iron based natural materials had been used as an electron acceptor in MIR to investigate the biodegradation of natural estrogenic compounds [231]. The biological degradation of estrogens under iron-reducing environment had been observed. MIR in coupling with organic oxidation was also examined in a continuous flow system, but the electron acceptor was in bulk phase [230]. Our experimental data shows that MIR mediated the biodegradation and transformation of CECs in a filtration system. There was different behavior among estrogens, pharmaceuticals, and antibiotics, while they were passing through the anaerobic FeOOH coated sand filter. For estrogen, E1 and E2 were mostly removed from the feeding water. However, ineffective removal of E3 was being observed in this study. The high removal of E2 was in agreement with previous study under the iron-reducing condition [231]. In the activated sludge, E1 and E2 were also effectively removed from the bulk solution [240]. Another observation also revealed that activated sludge system removed E3 greater than E2 and E1 ($E3 > E2 > E1$) [236]. Removal of E2 was approximately 47 % while the effluent concentration of E1 showed to have a higher than influent in a sewage wastewater treatment plant [245]. According to Ivanov et al. (2010), the removal of E2 was greater than E3 and E1 ($E2 > E3 > E1$) in the batch experiments mediated by MIR process [231]. It was addressed that E2 was primary transformed to E1 and 17- α -estradiol (α -E2) for different anaerobic conditions [246]. Thus, the removal of estrogens in wastewater treatment systems was varied and depended on microbial conditions and different treatment methods. Biodegradation and adsorption to activated sludge were proposed as primary mechanisms for removing estrogens [245].

Estrogens also have a tendency to adsorb onto stream and sediment biofilms [247]. The removal of E1 seems to be critical because it is a metabolite of E2. The results of this present study showed that the anaerobic iron coated sand filter was efficient in removing E1 and E2 even though E2 might be likely transformed to E1 during the treatment. However, the MIR did not remove E3 in a continuous flow system.

5.4.2 Pharmaceuticals

The experimental data in this present study showed that the anaerobic FeOOH coated sand filter effectively removed caffeine from the aqueous solution. Caffeine might be substantially used for electron donor in MIR. It was reported that caffeine was efficiently eliminated in the activated sludge wastewater treatment plant and anaerobic membrane bioreactor [248, 249]. According to Miao et al.(2005), caffeine was primarily adsorbed to biosolids in the activated sludge process and then degraded by aerobic microorganisms in the treatment system [248]. The removal of caffeine was also addressed in a biosand filter. The system removed more than 50 % of the caffeine in the laboratory scale [241]. The efficient removal of caffeine in this study was in agreement with a previous study in which more than 99 % of the caffeine was removed from the wastewater [250]. The lower portion of the filter was playing a significant role in removing the caffeine. A previous study reported that dehydrogenase was a novel enzyme to degrade caffeine [251]. Analysis of sand samples showed that enzymatic activities were highly detected along the filter depth. This evidence supports the assumption that caffeine was biodegraded in iron coated sand filter.

In contrast to caffeine, CBZ and GFZ appeared to have little removal in the anaerobic iron coated sand filter. The results were not surprising because these two selected pharmaceuticals are known as the biodegradable resistant compounds. Numerous studies revealed that little or no CBZ removal had been observed in sewage wastewater treatment plants [248, 252], anammox process [253], or bio-sand filter units [241, 250]. However, another study showed that CBZ was highly removed in a series aerobic and anaerobic membrane bioreactor system, and adsorption and biodegradation were proposed to play significant role in removing CBZ, but it was unclear and not yet verified [249]. In comparison to the bio-sand filters in previous studies [241, 242], the anaerobic iron coated filter system demonstrate a significantly better removal of CBZ, even though the removal rate was decreased over time. There could be a very slow biodegradation rate of this refractory compound under an iron-reducing environment in a continuous flow system. The results of a previous study showed that the lower infiltration rate results in a better removal of CBZ in the biosand filter [250].

Similarly, anaerobic iron coated sand showed little or no removal of GFZ. The removal efficiency of GFZ was decrease over time. Our data suggested that GFZ was unlikely degraded in the iron coated sand filter. This result was in contrast to data obtained in previous bio-sand column study in which approximately 94 % of CFZ was removed from drinking water [241]. However, another study showed that GFZ was not removed in an upflow bio-sand columns [242]. The results indicated that the anaerobic FeOOH coated sand filter was not an efficient means to remove GFZ from wastewater.

5.4.3 Antibiotics

A wide range of triclosan removal efficiency has been observed in the secondary treatment system. According to Onesios et al. (2014), triclosan was removed up to 90 % in a laboratory scale sand filter [242]. Triclosan was also effectively biodegraded in anammox process [253]. It was also addressed that antibiotics strongly absorbed into biomass and stream biofilms [253, 254]. Cologgi et al. (2014) documented that microbial film formation during MIR played a significant role in immobilizing and reducing toxic metals [89]. In this present study, we proposed that biofilm growth on the iron coated sand may also serve as the robust sorbent for antibiotics and biodegrade the absorbed compounds in the filtration process.

5.5 Conclusions

This study is the first to show the removal of CECs in a cost effective iron coated sand filter for water reclamation. The system efficiently removed E1, E2, caffeine, and antibiotics from the artificially reclaimed water. However, carbamazepine, gemfibrozil, E3 appeared to have a little reduction. Biodegradation and absorption to biofilm are assumed to play a role in removing CECs. The information obtained in this study may be useful for understanding the fate and transport of CECs in the subsurface and sedimentary environment where MIR is predominant.

CHAPTER 6. CONCLUSIONS

Although reclaimed water is promising as an alternative source for dealing with the current water scarcity on the globe, its long-term applications of inadequate treatment exhibits potential health risks and environmental problems. For safety use, reclaimed water must be free of any concerned substances or organisms. Low-cost technologies are a possible option for water reclamation in the context of efficiency, cost-effectiveness, and sustainability. Not only is it good for the environment, but it also costs less and more sustainable than other methods. For example, the biosand filter capability and efficiency to remove various pollutants has underpinned the deployment in different areas including wastewater disinfection and aquaculture and horticulture storm-water purification. In addition to the biological mechanism, most recent improvement of the soil based filtration is to increase physicochemical interaction and absorption for bacteria removal. Researchers have found that both bacterivory and electrostatic interaction enhance the quality of effluent water via a filtration process.

This study focused on: (1) the integrating interactions of grazers and adsorbent; and (2) the microbial activities in iron-reducing environments in both microcosms and filtration process in removing contaminants in agricultural and domestic wastewaters. The following section is the summary of findings throughout this study and suggestions for further research.

6.1 Capture and destruction of bacteria in the iron-rich natural soil filter

Although soil filtration has been extensively investigated as an onsite wastewater treatment method, the iron oxide-rich soil may become proper media to retain bacteria. The results of this chapter showed that Leilehua soil filter remove a high fraction of *E. coli* from the influent. Native soil protozoa digested most of the retained bacteria. This section provides additional useful information to understand the fate and transport of bacteria through sub-surfaces that are rich in iron oxides. Although this chapter has shown that the iron-rich soil greatly capture the *E. coli*, additional research may still need to obtain confident clues for bacterial removal in the iron-rich soil. Understanding the adsorption capability of iron-rich soil will promote optimized filter design and operation to create more efficient and environmentally sustainable filters for agricultural wastewater application.

6.2 Bacterial inactivation in iron-reducing environment

MIR is dominant in the subsurface and sedimentary environments. This chapter presents a new insight of in situ bacterial inactivation in the microcosm iron reducing environment. The results of this section showed that MIR process enhanced the inactivation of fecal bacteria. The MIR acted as an indirect mechanism to inactivate the fecal bacteria by producing of Fe^{2+} as an agent to facilitate the inactivation. Moreover, the inactivated bacterial cells becoming a source of electron donor to promote the MIR is an interesting finding in this study. An interesting bacterial inactivation mechanism found in this chapter would aid our knowledge of the survival of fecal bacteria in natural sedimentary and subsurface environments where the MIR process is prevalent. Further

researches may be needed to elucidate MIR for the enhancement of pathogen removal in natural and engineering processes. The future research should focus on the inactivation of other bacterial species and viruses. Obtain more knowledge of bacterial decay in MIR will help to improve the reclaimed water via storm water purification facilities, artificial aquifer recharge, and low-cost soil based water reclamation.

6.3 A novel filtration system to remove pathogenic bacteria from reclaimed water

Chapter 4 and 5 presents the performance of iron coated sand filter promoting MIR for removal of bacteria and CECs in reclaimed water. It was found that the column reduced the high level of *E. coli*, *E. faecalis*, *S. Typhimurium*. The anaerobic filter entirely removed *E. faecalis* from the influent. The integration anaerobic and aerobic filtration enhances the total removal of bacteria in the artificially reclaimed water remove ferrous production in effluent. This novel treatment configuration had shown a very efficient treatment means to eliminate the bacteria from the influent and organic substrates. It was also found that the biofilm of MIR had high adsorption affinity to *E. coli* cells.

This study was the first to investigate cost effective iron coated sand filter for water reclamation to remove CECs. It was found that estrogens, antibiotic, and caffeine had been mostly degraded microbial iron reduction in a continuous flow system. CBZ, GFZ, E3 appeared to be resistant to biodegradation under MIR. The results of these chapters provide useful information to understand the fate and transport of bacteria and CECs in the porous media in the subsurface and sedimentary environments where MIR

is predominant. However, future research will be needed to investigate an actual wastewater. More bacterial species should also be tested for the removal capacity of this such system. A sediment cover layers using the iron-based material should also be addressed to study the degradation of aromatic organic compounds in the sedimentary environments.

APPENDIX

Table A.1. Slow sand filtration installation in the United States (adapted from Graham (1988) [29].

State	Water Utility	Capacity, m ³ /d	Year
Idaho	Mission Creek	270	1964
	Skin Creek	330	1966
	Paradise Valley 1	270	1967
	Paradise Valley 2	270	1967
	Fernwood	530	1967
	Monrovia	110	1968
	Twenty Mile	45	1968
	Beeline	570	1968
	East Hope	950	1975
	City of Salmon	11,000	1976
	Yellow Pine	160	1976
	City of Challis	5,300	1981
	Colburn	830	1985
	Schweitzer Basin	120	1985
	Rocky Mountain Academy	110	1986
	Cavanaugh Bay	160	1988
Rockford Bat	3,300	1988	
Harbor View Estates	1,100	1988	
Sunnyside	160	1988	

Oregon	Salem	150,000-170,000	1958
	Salem	190,000-260,000	1970
	Stayton	15,500	1975
	Stayton	15,500	1978
	Westfir	540	1986
	Wickiup Water District	650	1987
	Cape Meares	380	1988
	Detroit	1,100	1988
	Idanha	1,100	1988
	Kernville	2,300	1988
	Oaklodge Water District	11,400	1988
	Panther Creek Water District	760	1988
	Astoria	3,800	1988
Washington	Blue Spruce Water District	910	1987
	Eatonville	3,300	1988
	Doe Bay Orcas Island	760	1988
	Cashmere	14,200	1988
Colorado	Empire	950	1984
Vermont	McIndoe Falls	82	1974
New York	Waverly	4,500	1982

Table A.2. The known IRB species based on 16S rRNA analysis

Species	References
<i>Geobacter metallireducens</i>	[70, 71]
<i>Geobacter sulfurreducens</i>	[70, 71]
<i>Geobacter hydrogenophilus</i>	[71, 255]
<i>Geobacter chappellei</i>	[71, 255]
<i>Geobacter grbiciae</i>	[255]
<i>Geobacter toluenoxydans</i>	[256]
<i>Geobacter humireducens</i>	[71]
<i>Shewanella oneidensis</i>	[86]
<i>Shewanella aga</i>	[71]
<i>Shewanella putrefaciens</i>	[71]
<i>Shewanella potomacii</i>	[71]
<i>Shewanella arcitica</i>	[257]
<i>Desulfuromonas axetecigens</i>	[71]
<i>Desulfuromonas palmitatis</i>	[70, 71]
<i>Desulfuromonas acetoxidans</i>	[70, 71]
<i>Desulfuromusa succinoxidant</i>	[70, 71]
<i>Desulfuromusa kysingii</i>	[70, 71]
<i>Desulfuromusa bakii</i>	[70, 71]
<i>Pelobacter acidigallici</i>	[70, 71]
<i>Pelobacter venetianus</i>	[70, 71]
<i>Pelobacter acetylenicus</i>	[70, 71]
<i>Pelobacter carbinolicus</i>	[70, 71]
<i>Pelobacter propinocus</i>	[70, 71]
<i>Geovibrio ferrireducens</i>	[71, 258]
<i>Geothrix fermentans</i>	[72] [71]
<i>Desulfitobacterium aromaticivorans</i>	[256]
<i>Desulfitobacterium autotrophicum</i>	[71]
<i>Desulfovibrio desulfuricans</i>	[71]
<i>Desulfovibrio vulgaris</i>	[71]
<i>Desulfovibrio hydrogenophilus</i>	[71]
<i>Desulfovibrio propionicus</i>	[71]
<i>Clostridium pasteurianum</i>	[71]
<i>Bacillus infernus</i>	[71]

<i>Roddobacter capsulatus</i>	[71]
<i>Thiobacillus thiooxidans</i>	[71]
<i>Thiobacillus ferrooxidans</i>	[71]
<i>Ferrimonas balearica</i>	[71]
<i>Geospirillum barnesii</i>	[71]
<i>Wolinella succinogens</i>	[71]



Figure A.1. The Multi-Soil-Layer filter system



Figure A.2. The microcosm MLS system



Figure A.3. MIR microcosm setup for using *E. coli* as a source of electron donor



Figure A.4. FeOOH coated sand column at initial time



Figure A.5. MIR in FeOOH coated sand as indicated by darken color

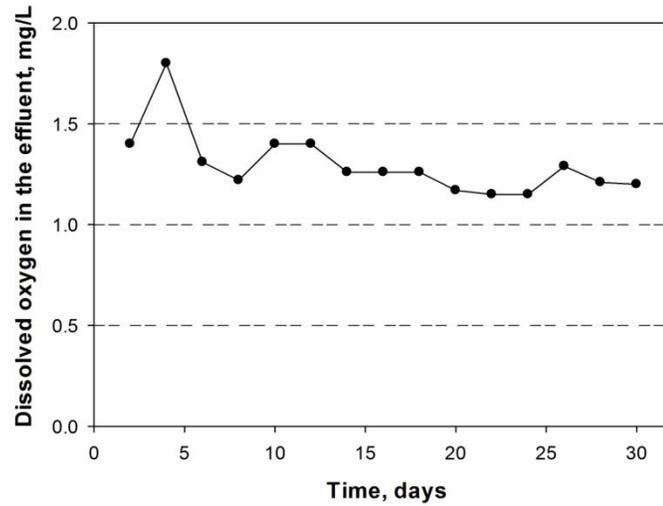


Figure A.6. The dissolved oxygen concentration in the effluent during the first 30 days startup period.

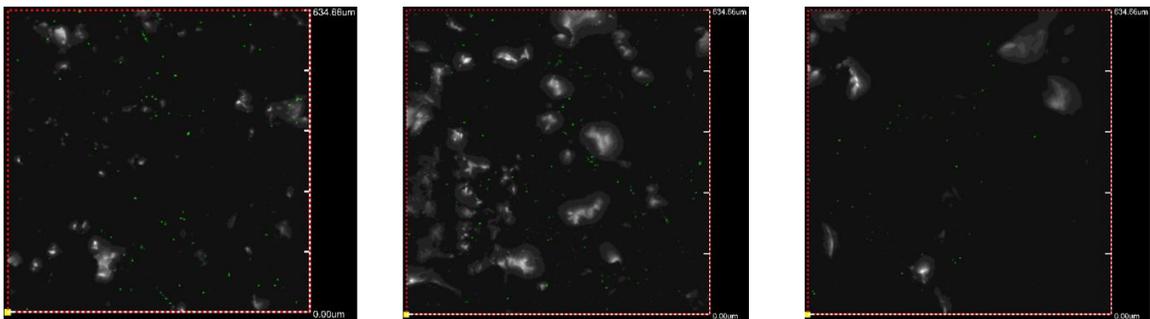


Figure A.7. Attachment of *E. coli* ATCC29522 to biotic and abiotic surfaces. Top view 3D micrographs selected from 3D view in Z-stack from the bottom to the top. A) IRB biofilms, B) iron oxide surface, C) Magnetite surface.

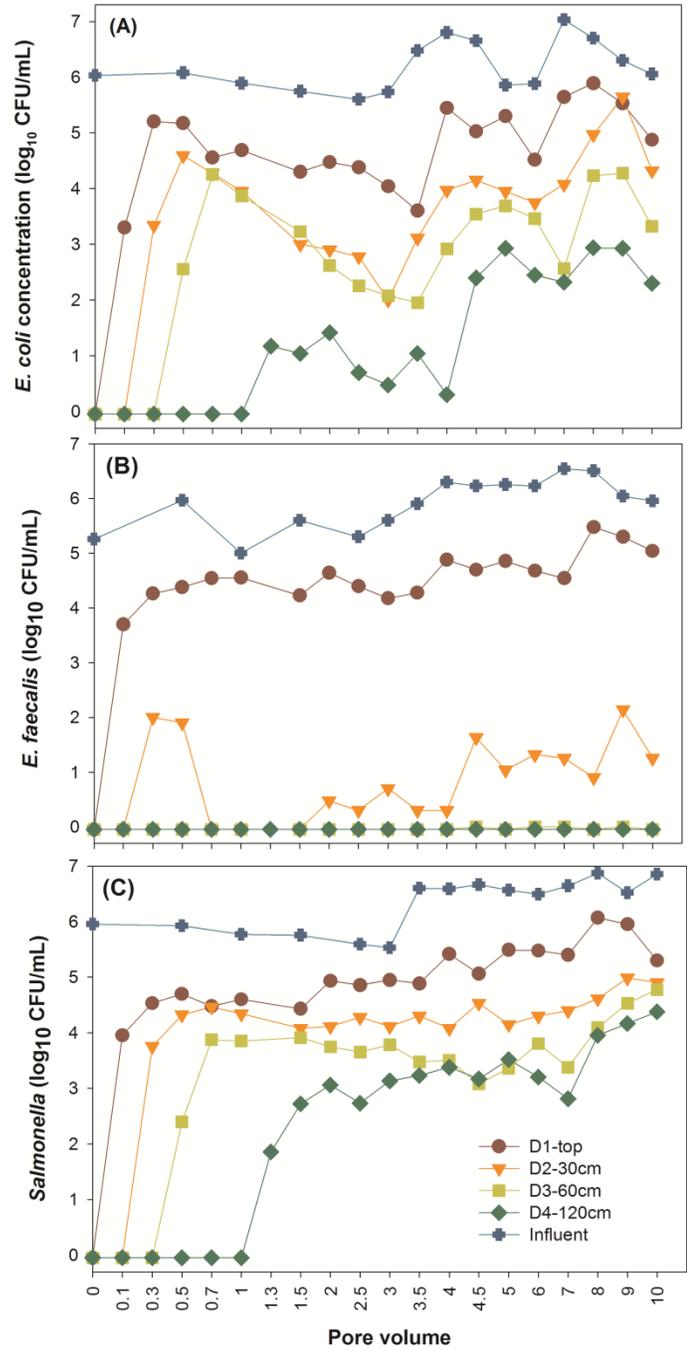


Figure A.8. Concentrations of *E. coli* (A), *E. faecalis* (B), and *Salmonella* Typhimurium (C) in the influent, at different column depths (D1, D2, D3, and D4), and effluent.

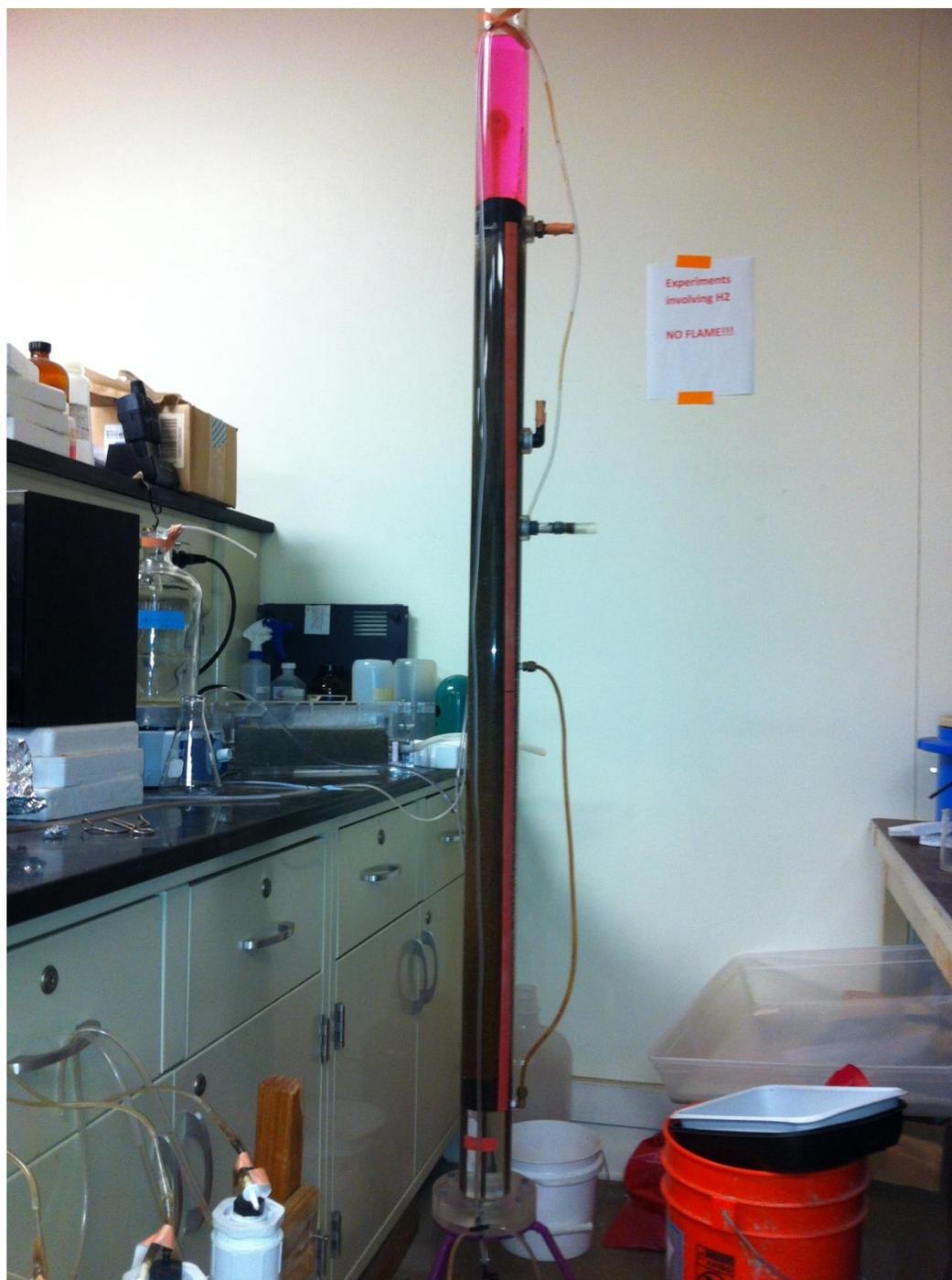


Figure A.9. Resazurin used as a redox indicator. Clear color at the bottom indicates the oxygen limit in the column.



Figure A.10. The horizontal aerobic filter



Figure A.11. Precipitation of ferric oxides in the aerobic filter.

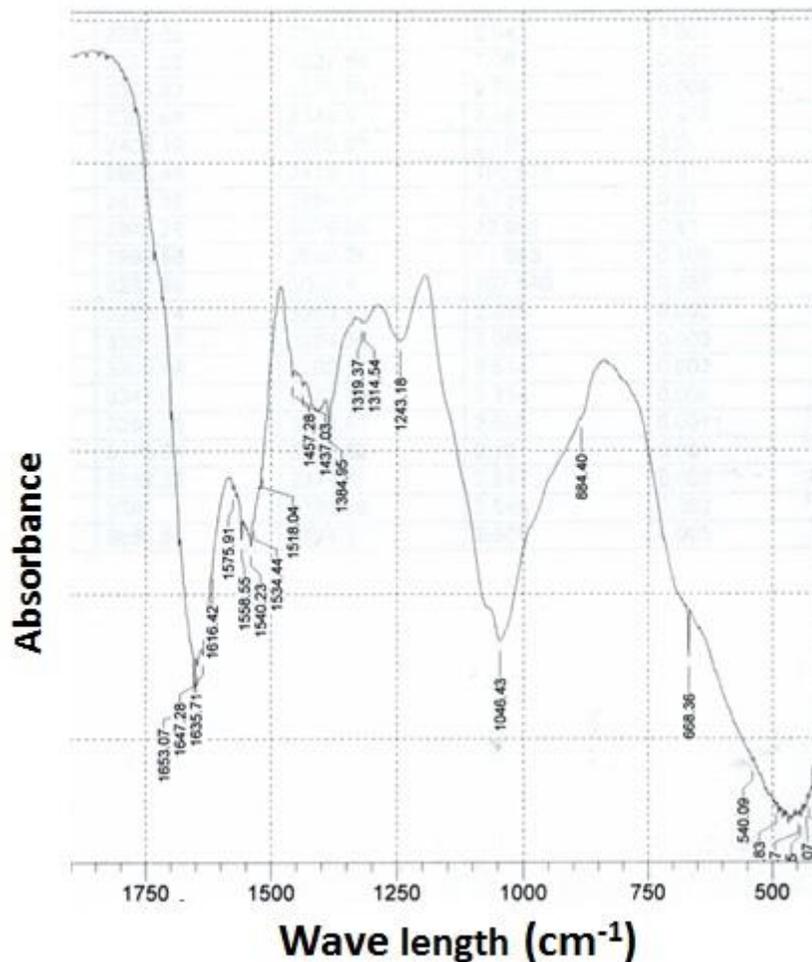


Figure A.12. Detection of iron oxide formed during aeration process by FTIR analysis.

Salmonella Shigella (SS) Agar Verification for *S. Typhimurium* quantification

1. Experimental procedure

Culture preparation: *E. coli*, *E. faecalis*, and *S. Typhimurium* were grown in LB broth overnight to a steady state (OD₆₀₀ 1.9, 1.4, and 1.6). 1.0 mL of each culture was extracted and washed in 1.0xPBS for three times. The washed cells were suspended in 1.0xPBS. A serial dilution was made by transferring 1.0mL to a vial containing 9.0mL of

sterile PBS. 2.0mL of each culture at the same dilution level was transferred to a sterile test tube to form the 6.0 mL mixed culture. Triplicate of mixed culture was prepared.

1.0mL of an appropriate dilution of mixed culture and pure culture was filtered through a 0.45µm membrane. The filtered membranes then placed onto mTEC, mEI, and SS agar plates to selectively identify *E. coli*, *E. faecalis*, and *S. Typhimurium*, respectively. The incubation procedure was at 35°C for 2 hours and subsequent 44.5°C for 22 hours for *E. coli*, 41°C for 24 hours for *E. faecalis*, and 35°C for 24 hours for *S. Typhimurium*.

2. Results and discussion

The result showed that Salmonella grew selectively on SS agar with black color colonies. The numbers Salmonella in triplicate mixtures were approximately equal to 1/3 of pure culture. Figure 1 showed that there were no either *E. coli*, or *E. faecalis* grew on SS agar. The numbers of *E. coli* and *E. faecalis* in mixed culture was likely equal to 1/3 of pure culture. The results suggested that each species only grew on selective agar plates. Thus, SS agar is a valid method that can be used to quantify *S. Typhimurium* in water samples.

Table A.3. The concentration of *S. Typhimurium*, *E. coli*, and *E. faecalis* in mixed and pure culture.

Samples	<i>S. Typhimurium</i> (CFU/mL)	<i>E. coli</i> (CFU/mL)	<i>E. faecalis</i> (CFU/mL)
Geomean+STD	$1.06 \times 10^9 \pm 8.02 \times 10^7$	$8.53 \times 10^8 \pm 4.04 \times 10^7$	$3.32 \times 10^8 \pm 3.51 \times 10^7$
Pure culture	3.7×10^9	3.6×10^9	8.1×10^8

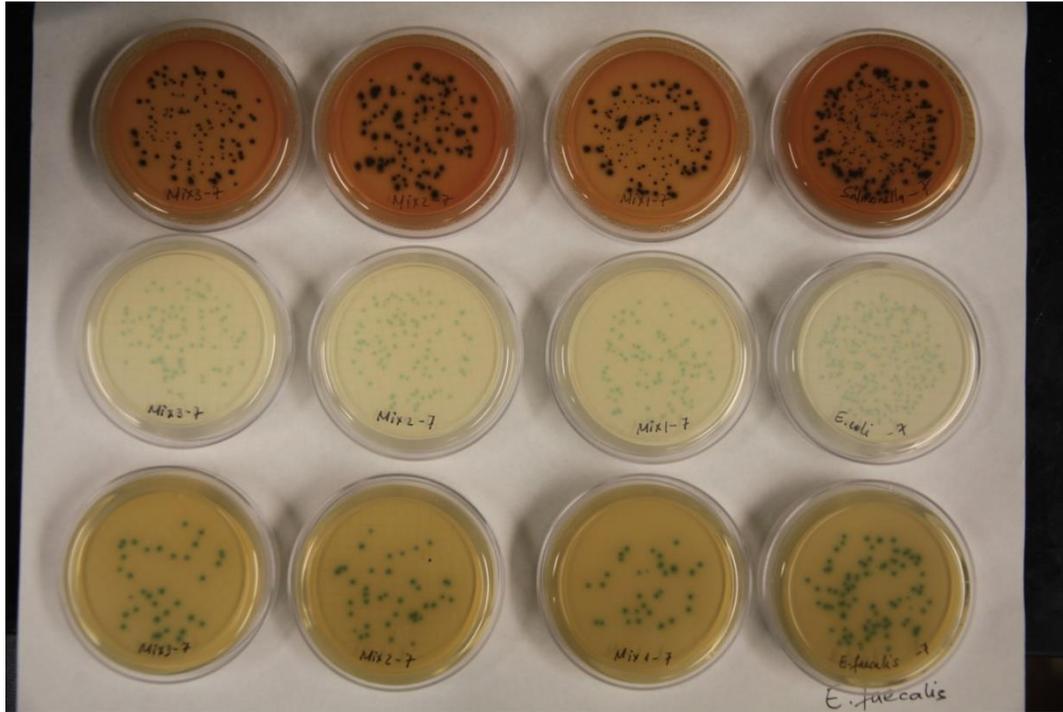


Figure A.13. Colonies of *S. Typhimurium* on SS agar plates and *E. coli* and *E. faecalis* on mTEC and mEI agar plates.

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