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INFLUENCE OF COMPOST PARTICLE SIZE ON PATHOGEN SURVIVAL UNDER GREENHOUSE CONDITION

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ABSTRACT

The number of foodborne pathogen outbreaks related to fresh produce has increased significantly in recent decades. Animal waste directly applied to agricultural field is one of the possible contamination sources for fresh produce. Composting is one of the recommended means for waste treatments to eliminate or reduce pathogens in manure on farms. Although pathogens can be eliminated by proper composting process, pathogens are able to survive, recolonize and regrow on compost heap surfaces under certain conditions. Due to the outdoor nature of composting process, bioaerosols with different particle sizes can be generated on compost surfaces which can carry pathogens, travel via air and contaminate fresh produce field nearby. This study was to investigate the survival of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in dairy compost with different particle sizes as affected by initial moisture content and seasonality under greenhouse conditions. Also, in order to understand the pathogen survival strategies, microscopic observations of the interactions between bacterial cells and compost particles were explored using several approaches.

The mixture of *E. coli* O157:H7 and avirulent *S.* Typhimurium strains were inoculated into the finished composts with initial moisture content of 20, 30 and 40%. Then, the finished compost samples were sieved into three portions with particle sizes of >1000, 500-1000 and <500 μm, and stored in greenhouse for 30 days. At selected intervals, compost samples were tested for pathogen population. For the microscope study, the morphological and surface characteristics of compost particles with different particle sizes were analyzed by a surface profiler. Then the green fluorescence proteins

(GFP)-labeled *E. coli* O157 inoculated compost samples were observed under a fluorescence microscope, followed by spectral analysis and unmixing. Our next approach was to use immunofluorescence (IF) protocol for enhancing the GFP signal with different fluorescence dyes, such as AlexaFluor 594 and quantum dots.

For the greenhouse study, the moisture contents in compost samples dropped rapidly to under 10% within 5 days of storage followed by gradual decline till 30 days in all treatments. For compost with moisture contents of 20 and 30%, the average *Salmonella* reductions in compost with particle sizes of >1000, 500-1000 and <500 μm were 2.15, 2.27 and 2.47 log CFU g-1 within 5 days of storage in summer, respectively, as compared with 1.60, 2.03 and 2.26 log CFU g-1 in late fall, and 2.61, 3.33 and 3.67 log CFU g^{-1} in winter, respectively. For samples with initial moisture content of 40%, the *Salmonella* reductions in compost with particle sizes of >1000 and <1000 μm were 2.14 and 3.17 log CFU g^{-1} within 5 days in summer, respectively, as compared with 3.17 and 3.16 log CFU g^{-1} in late fall, and 2.93 and 3.36 log CFU g^{-1} in winter, respectively. For compost with moisture contents of 20 and 30%, the average *E. coli* O157 reductions in compost with particle sizes of >1000, 500-1000 and <500 μm were 1.98, 2.30 and 2.54 log CFU g^{-1} within 5 days of storage in summer, respectively, as compared with 1.70, 2.56 and 2.90 log CFU g^{-1} in winter, respectively. For samples with initial moisture content of 40%, the *E. coli* O157 reductions in compost with particle sizes of >1000 and <1000 μm were 2.08 and 2.48 log CFU g^{-1} within 5 days in summer, respectively, as compared with 2.20 and 2.84 log CFU g^{-1} in winter, respectively.

In fluorescence microscope observation, the sensitivity is negatively affected by broad spectra and high intensity from autofluorescence of compost matrix. For spectra analysis, the emission wavelength and intensity of both GFP and autofluorescence from compost were measured and compared statistically, however the method could not provide information about location of individual cells in compost matrix visually. Additionally, IF results showed that GFP in *E. coli* O157: H7 and autofluorescence of compost can be differentiated both visually and quantitatively according to the signal intensity. However, Qdots results showed that it is an more effective treatment for enhancing signals of GFP-labeled *E. coli* O157 in compost matrix compared with AlexaFluor based IF method.

Our results revealed that compost with larger particle size supports pathogen survival better than the compost with small particle size, and the initial rapid moisture loss in compost may contribute to fast inactivation of pathogens in the finished compost. Green fluorescence protein (GFP) expressed by bacterial cells is not sufficient to differentiate target cells from background autofluorescence in compost. Spectra analysis can separate overlap emissions from GFP-labeled pathogen and compost statistically, however, amplification of GFP signal by immunofluorescence methods, esp. Qdots, can improve the detection of target cells.

DEDICATION

I would like to dedicate this work to my mother, Lingping Zhang, my father, Ke Diao. Without their support, and encouragement this could not have been possible.

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CHAPTER ONE

LITERATURE REVIEW

Introduction

In the past decade, agricultural production is the second highest sector in the national economy. Workers in the agricultural field, which count for 1 out of 12 job opportunities, produce food for consumption up to 86% in total (USDA, 2011).

Meat and fresh produce as agricultural products can be contaminated by pathogenic microorganisms. Those human pathogens that are considered as foodborne are usually introduced in food during the growing and processing steps. Therefore, preventing and controlling these pathogens is critical to guarantee food safety. In the growing step, livestock and poultry can take up pathogens from their feed, drinking water and the environment. Pathogens can be shed in the feces of healthy or sick animals, and then contaminate food via soil, irrigation water and fertilizer. Application of animal manure to agricultural land is a widely used method to improve the soil fertility, such as nitrogen, phosphorous and potassium, and physical properties of soil, such as structure, permeability and water holding capacity. Due to the presence of foodborne pathogens in animal waste (Islam, 2005), this practice can result in the contamination of food crops which grow in the manure amended soil (Oliveira et al., 2012). To ensure the biological safety of these types of foods, it is critical that pathogens are eliminated or properly controlled in animal wastes.

Foodborne Illnesses Associated with Fresh Produce

According to the Centers for Disease Control and Prevention (CDC), there were a total of 48 million foodborne illnesses, 128,000 hospitalizations and 3,000 deaths associated with foodborne pathogens in the United States each year (CDC, 2011). Most (58%) of the foodborne outbreaks are caused by Norovirus (Scallan et al., 2011), followed by 9 well-known foodborne pathogens including *Escherichia coli* O157: H7, *Salmonella* spp., *Listeria monocytogenes*, *Campylobacter*, *Shigella*, *Vibrio*, *Yersinia*, *Cryptosporidium* and *Cyclospora*. Nontyphoidal *Salmonella spp.* is the leading cause of hospitalizations and deaths (Scallan et al., 2011). Among food items most frequently associated with the outbreaks are seafood, fresh produce, poultry, beef and pork.

The number of foodborne illness outbreaks related to fresh produce has increased significantly in recent decades (CAST, 2009). From 1973 through 1997, a total of 190 produce-associated outbreaks, 16,058 illnesses, 598 hospitalizations, and 8 deaths were reported (Sivapalasingam et al., 2004). However, from 1990 through 2003, there were 554 foodborne illness outbreaks and 28,315 cases related to fresh produce in total (DeWaal et al., 2006). A few years ago, leafy greens were the main source of produce related outbreaks. In September 2006, at least 199 people from 26 states were infected by *E. coli* O157: H7 and 3 deaths were attributed to this fresh spinach related foodborne outbreak (CDC, 2006). The *E. coli* O157: H7 outbreak strain was suspected to have been transmitted from the nearby cattle ranch to the spinach field. In 2011, a multistate outbreak of *L. monocytogenes* infection caused 147 cases of illnesses and 33 deaths across 28 U.S. states due to contaminated cantaloupes. The origin of the pathogen

remained unknown; however the truck and processing equipments were the suspected vehicles for pathogen transmission. Also, it was noted that the lack of a pre-cooling step to remove field heat from the cantaloupe before cold storage could facilitate pathogen growth. This is considered the worst food-related outbreak in U.S. history in terms of the number of deaths (CDC, 2012).

To improve fresh produce safety, "Good Agricultural Practices" (GAPs) are widely recognized and applied in the field. According to the Food and Agriculture Organization (FAO, 2002), GAPs consist of following elements about on-farm processing and storage: (1) Harvesting food products should follow the pre-determined pre-harvest intervals and withholding periods. (2) When processing fresh products on farms, the environment and products should be free from contaminants. (3) For washing, the recommended sanitizers and pathogen-free water should be used. (4) Food products should be stored under hygienic and appropriate environmental conditions. (5) Food products should be packed for transport from the farm in clean and appropriate containers. Also, the GAPs include the guidelines for crop growing: (1) Understand the characteristics of cultivars and varieties. (2) Devise crop sequences in order to enhance soil biological and chemical quality. (3) Use organic and inorganic fertilizers in balanced time and quantity. (4) Recycle crop and other organic residues. (5) Rotate livestock into crop and pasture rotations. (6) Adhere to safety regulations for the operation of equipment and machinery (FAO, 2007). These practices ensure that agricultural products are produced with better safety properties. Food Safety Modernization Act (FSMA) was signed into law by President Obama on January 4th, 2011. The significance of the act is the focus of federal

regulators shifted from responding to contamination to preventing it (FDA, 2011). The FSMA will establish standards for produce safety from growing, harvesting, post-harvest handling to packing to ensure the safety of fresh produce for human consumption. More mandated inspections with preventive controls in place are required (FDA, 2011).

E. coli O157: H7

E. coli is a Gram-negative, rod-shaped, facultative anaerobic, highly adaptable organism which belongs to the family of *Enterobacteriaceae*. *E. coli* strains are one of the predominant enteric species in human intestinal tract. They are classified by its antigens: O for lipopolysaccharides (LPS), H for flagellum and K for capsule (Pexara, 2012). There are hundreds of serotypes indentified so far. Although most of the *E. coli* strains are harmless to humans, there is a small group of strains recognized as Shiga-toxigenic *E. coli* (STEC). They can produce Shiga toxins that can cause serious illnesses. They are mainly divided into six pathogenic groups: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC). Of these, only the first 4 groups have been implicated in foodborne illness. The main symptoms are hemorrhagic diarrhea, dysentery and hemolytic uremic syndrome (HUS). *E. coli* O157: H7, O140, O26 are examples of STEC.

E. coli O157: H7 causes an estimated 73,480 illnesses, 2,168 hospitalizations and 61 deaths in the United States annually (Mead, 1999). It is classified as EHEC. It was first recognized as a foodborne pathogen in 1982 (Riley et al., 1983). This serotype can be

found in animal intestinal tracts, but it usually associates with ruminant animals due to the lack of receptor for the toxin it produces (Pexara, 2012). *E. coli* O157: H7 can produce Shiga toxins, known as stx1 and stx2, and these shiga toxins can block mRNA translation and cause cell death. Both the Stx1 and Stx2 contain a single enzymatically active A subunit and several B subunits. If the cell has the receptor (globotriaosylseramide) for the Stxs, the toxins are able to bind to the cell, followed by internalization to the cytoplasm. Once the Stxs are in the host cells, the A subunit of them can bind to the 28s rRNA and release an adenine residue. The mRNA translation and the protein synthesis are inhibited. At the same time, the B subunits are responsible for binding to the neutral glycolipid receptors (Jay, 2000). However, Stx2 is considered to be more significant than Stx1 in causing foodborne illnesses.

Ground beef, fresh vegetables, fruits and apple cider are the types of food commonly contaminated with *E. coli* O157: H7 and cause foodborne illness outbreaks. For example, in 2007, 21.7 million pounds of Topp's brand frozen ground beef patties were recalled due to the *E. coli* O157: H7 contamination. In 2009, there were recalls of 41,280 pounds and 545,699 pounds of beef products from JBS Swift Beef Company and Fairbank Farms, respectively. The spinach outbreak in 2006 resulted in 31 HUS and 3 deaths due to contamination of *E. coli* O157: H7 (CDC, 2006).

Although *E. coli* O157:H7 accounts for ca. 75% of the EHEC infections, other non-O157 EHEC foodborne illnesses are becoming of increasing concern. *E. coli* O26, O111, O103, O121 and O45 are the most frequently non-O157 serotypes associated with foodborne illnesses (Mathusa et al., 2010).

Salmonella spp.

Like *E. coli*, *Salmonella* is a Gram negative, rod-shaped, facultative anaerobic and predominant motile bacterium from the family of *Enterobacteriaceae*. It was discovered by Dr. Salmon from the intestine of a pig in 1885.

The nomenclature of *Salmonella* was originally based on clinical considerations, such as *S.* Typhi which cause typhoid fever in patient. The taxonomy of it has greatly changed based on DNA-DNA hybridization and multilocus enzyme electrophoretic characterizations (Jay, 2000). Now, all *Salmonella* have been placed into two species, *S. enterica* and *S. bongori*. *S. enteric,* which is a great public health concern, includes six subspecies: *enteric* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indica* (VI). Now, *Salmonella* spp. are commonly referred to by their serotype names. For example, *S.* Typhimurium is the short form for *Salmonella enterica* subsp. *enteric* serotype Typhimurium (Hammack, 2012).

Some *Salmonella* serotypes are highly adapted to humans, like *S.* Typhi and *S.* Paratyphi A, which cause typhoid fever, a severe human disease with a mortality rate of about 10%. Symptoms include high fever, lethargy, abdominal pains, diarrhea, headache, achiness and loss of appetite. In this paper, the nontyphoidal Salmonellosis, caused by serotypes other than *S.* Typhi and *S.* Paratyphi A with a mortality rate of lower than 1%, is mainly discussed. The infectious dose for *Salmonella* infection can be as low as under 10 cells. Symptoms include nausea, vomiting, abdominal cramps, diarrhea, fever and headache. There are over 2,500 different *Salmonella* serotypes but the serotypes mostly linked to human nontyphoid Salmonellosis are: *S.* Enteritidis, *S.* Typhimurium and *S.*

Newport. According to the recent estimation from CDC, It causes about one million cases annually in the U.S. (Hammack, 2012).

Salmonella is widely dispersed in nature. It is primarily present in the intestinal tracts of birds, reptiles, farm animals and humans. Thus, the pathogens can be spread to the environment via fecal routes (Jay, 2000). *Salmonella* can also persist in environments for an extend period of time (Hammack, 2012), and subsequently contaminate irrigation water, equipments and food related surfaces.

The mechanisms of *Salmonella* infection are not well understood. Although people have already indetified an enterotoxin and a cytotoxin in pathogenic *Salmonella* serovars, they are not believed to be effective in the gastroenteritis syndrome (Jay, 2000).

Eggs, poultry, meat and dairy products are most commonly contaminated with *Salmonella* spp.. Additionally, fresh produce has also recently emerged as the source of *Salmonella* outbreaks (Hammack, 2012). In 2010, a multistate outbreak of *S.* Enteritidis infections associated with shell eggs resulted in 1,939 illnesses (CDC, 2010). In 1985 and 1994, *Salmonella* outbreaks associated with dairy products involved more than 200,000 and 224,000 people, respectively. Tomatoes have been reported as a food source of a *S.* Typhimurium outbreak in 2006. This outbreak involved 183 cases in 21 states (CDC, 2006).

Pathogens in Animal Manure

As the global population increases, intensive animal and livestock production increases as well. This leads to increasing generation, accumulation, and disposal of large

amounts of animal wastes around the world. The USDA estimated that over 500 million tons of manure is produced annually by livestock and poultry operations (EPA, 2009). Manure consists of animal excreta (feces and urine), bedding, dilution water and other animal body excreta. Slurry however contains only animal excreta but not a lot of bedding materials (Pell, 1997). Manure is occasionally mixed with water into ponds, and the slurry is later spread onto fields (Himathongkham et al., 2000).

Because human pathogens are present in animal intestinal tracts, it is not surprising that animal manures can contain pathogens. McLaughlin et al. (2012) reported that zoonotic pathogens such as *Campylobacter*, *Listeria*, and *Salmonella* were present in swine manure lagoon water year-round, but the population changed by seasons. Semenov et al. (2007) reported that *E. coli* O157: H7 and *S.* Typhimurium were suppressed by background microorganisms in manure. They developed a predictive model for pathogen survival in manure. For *E. coli* O157:H7, the survival time in manure ranged from <7 days at 33 ℃ to 159 days at 7 ℃, and the survival time for *S*. Typhimurium ranged from 227 days to <21 days at 7 ℃ and 33 ℃, respectively. In addition, *E. coli* O157: H7 and *Salmonella* have a more rapid die-off rate in poultry manure samples than in cow manure (Himathongkham et al., 1999). However, Payne et al. (2007) found if other conditions are desirable poultry litter with a water activity of 0.96 or higher will allow the growth of *Salmonella*.

In conclusion, pathogens are able to survive in animal manures and have the potential to transmit from manure to fresh produce. Thus, direct application of manure to land is not recommended.

Organic Fertilizers and Manure-Amended Soil

Organic fertilizers are widely used in agricultural production in the United States, including animal manure, slurry, compost, bloodmeal, bonemeal, humic acid, amino acids, seaweed extracts and others (Sullivan, 2001). Although the nutrient density in them is lower than that in inorganic fertilizers, using organic fertilizers still has many advantages. Organic fertilizers can release nitrogen (N), phosphorus (P) and potassium (K) at a consistent rate, have less nutrient loss, help to retain soil moisture, keep the soil structure, retain nutrients in a plant-accessible form and are chemical-waste free (Shperber et al., 2011). The major disadvantages are higher price, lower nutrient content and excess amount of calcium and sodium (Shperber et al., 2011).

Animal manures are most commonly used as organic fertilizer (Watson et al., 2002). Due the presence of human and animal pathogens in fresh manure, safety and quality control of organic fertilizers are generally focused on animal manures and manure-based composts. Bacterial, fungal, viral, and parasitic organisms can transmit from manure-amended soil to produce (Diesch et al., 1969). It is necessary to have a certain time period after manure is incorporated into the soil before either planting or harvesting. According to USDA rules, during the growth, if the produce has direct contact with the soil, a minimum of 120 days interval is required, while for all other produce grown on soil, a 90 day interval is mandatory (USDA, 2012).

Besides animal manure, there are many other types of organic fertilizers. For example, animal by-products such as blood meal, feather meal, fish emulsion and bone meal are used as organic fertilizer as well. Plant by-products as organic fertilizers include

alfalfa meal, cottonseed meal, soybean meal and plant ashes (Baker et al., 2005). Additionally, mined minerals are the nutrient source in traditional and organic crop production.

Manure is not considered as an entirely safe material to use for growing fresh produce because the pathogens can survive in manure amended soil for several months. Islam et al. (2005) conducted a field study to determine the survival of *E. coli* O157; H7 on carrots, onions and contaminated soil. *E. coli* O157:H7 was detectable in manure-amended soil samples till 154-196 days, and survived on onions and carrots for 74 and 168 days, respectively. Their results suggested the possibility of pre-harvest contamination due to use of animal manure.

Indigenous microflora are an important factor affecting the survival of pathogens in manure amended soil. Jiang et al. (2002) determined the effects of autoclaved and unautoclaved sandy loam soils on the death of *E. coli* O157: H7 and *L. monocytogenes*. In their study in 2002, *E. coli* O157: H7 survived for up to 77, >226, and 231 days in autoclaved manure-amended soil at 5, 15, and 21 ℃, respectively. *L. monocytogenes* survived for up to 43, 43, and 14 days in autoclaved manure-amended soil at 5, 15, and 21 ℃, respectively. As compared to 43, 21, and 21 days at 5, 15, and 21 ℃, respectively, in unautoclaved manure-amended soil (Jiang et al., 2004). Rapid die off of *L. monocytogenes* in unautoclaved soil at growth temperature suggested that indigenous soil microorganisms expedite pathogen reduction in soil-manure mixture. McLaughlin et al. (2011) investigated the influence of background microbiota, temperature, moisture content and strain motility upon pathogen survival in soil samples. In non-sterile soil,

viable *L. monocytogenes* cell counts declined over a 6-day period, compared to an increase of over 1 log in the first 4 days in sterile soil. Growth of *L. monocytogenes* was observed in autoclaved soil samples due to the lack of competitive microflora.

Both moisture content and temperature are important factors that influence the survival of pathogens in soil. Rothrock et al. (2012) conducted a greenhouse experiment to determine the survival dynamics of *E. coli* O157: H7 in soil and they found that pathogens survived at a significant low level in 45% moisture soil samples as compared to 25% moisture soil samples. McLaughlin et al. (2011) reported that *L. monocytogenes* had comparable survival rates in soil samples in sealed and unsealed soil samples within the first week. However, desiccation may be influential to pathogen survival after prolonged time. They also revealed that temperature had major impact on pathogen survival in soil. *L. monocytogenes* survived significantly higher at 8 ℃ in soil than at 25 and 30 ℃ over a 4-day period. At 8 ℃, 10^3 CFU/g of *L. monocytogenes* were recovered up to day 14.

Due to the possibilities that manure and manure-amended soil can harbor pathogens which can further transfer to produce, animal manure needs to be processed to get rid of pathogens prior to land application. Composting is one of the recommended means for various types of manure treatments to reduce pathogens on the farms.

Composting Overview

Composting is the degradation of organic matter by microorganisms in defined composition of material, moisture content and method which result in the creation of a

nutrient rich soil amendment. Composting is a complicated process which can effectively reduce human pathogen in the material (Glanville et al., 1997). The final product of composting process is a nourishing, stable and humus-like material (Dickson et al., 1991). Compost is generally recommended as an amendment to soil in gardens, landscaping, horticulture, and agriculture.

Composting can be achieved by several methods, including outdoor windrow system, in-vessel system, passively or actively aerated static piles (Sherman et. al, 2005). Passive and static heaps are both stacking of composting materials into heaps. However, passive heaps are managed very little while static forced aeration or frequent mechanical turning are required for static aerated piles.

Composting process consists of 3 main phases and each phase includes different types of microorganisms (EPA, 1999). The first one is mesophilic phase. In this phase, temperature in compost increases as mesophilic microorganisms rapidly multiply. As a result of microbial metabolism, a production of organic acids leads to the decrease of pH (Hassen et al., 2002). The thermophilic phase begins when composting temperature reaches 50℃ to 70℃. In this phase, actinomycetes and thermophilic bacteria are most active. In addition during this phase, the oxygen level decreases (Herrmann et al., 1997). At last, as the bacteria activity gets low, composting process enters the maturation phase or cooling phase, when temperature goes down under 40℃. In this phase, there is a chance for the compost to get contaminated or the mesophilic bacteria to regrow (Kim et al., 2009).

Microorganisms play an important role in the composting process. Microorganisms such as bacteria, actinomycetes, fungi, protozoa and rotifers (Trautmann et al., 2010) can break down organic materials such as carbohydrates, proteins and fats to produce the compost in certain optimal environment. Bacteria are the most numerous microorganisms in compost which complete most of the denaturation process. Ishii et al. (2000) used Density Gradient Gel Electrophoresis (DGGE) to determine the microorganisms present in the composting process. They revealed that in mesophilic phase, fermenting bacteria, such as *Lactobacillus*, were present, whereas *Bacillus* dominated the thermophilic phase. During the cooling phase, the bacterial community was more complex than in the first two phases. Additionally, actinomycetes are mainly responsible for breaking down complex substances such as cellulose, lignin, chitin, and proteins, whereas fungi help to break down tough debris and let bacteria continue the decomposition process. Protozoa play a minor role in degradation of organic material. And rotifers can control bacteria and small protozoan populations (Trautmann et al. 2010).

To achieve the quality and microbiological safety of composting process, Environmental Protection Agency (EPA) and U.S. Department of Agriculture (USDA) have guidelines for composting. The 40 CFR Part 503 guidelines have been applied by EPA for pathogen reduction in biosolids. These rules regulate the safety and hygiene of biosolids. According to these guidelines, fecal coliform population should be under 1000 MPN g^{-1} and *Salmonella* should be under 3 MPN $4g^{-1}$ in compost on dry basis (EPA, 1999). The national organic program (NOP) of USDA set the standard for animal-manure composting handling in 205.203. Briefly, the initial carbon to nitrogen (C:N) ratio should

be between 25:1 and 40:1. The temperature of compost heaps should be maintained at >55℃ for aerated static piles or in-vessel systems for 3 days. For windrow systems, the temperature of the compost piles should be maintained at >55℃ for at least 5 days with at least 5 turnings within 15 days (Misra et al., 2003).

The major parameters of composting process are C:N ratio, temperature, moisture content and aeration. C:N ratio can affect the composting process greatly since the preferred ratio can support microorganisms to grow and replicate. Although the C:N ratio of 25: 1 to 40: 1 is recommended by NOP, a 25:1 - 30:1 ratio is preferred. Moisture content is another important parameter influencing composting process, since enough available water is essential for microorganisms to metabolize. The initial moisture content of compost mixture should be between 40% and 65%, however, 50% to 60% is preferred (Sherman, 2005). The pH of the composting materials is another factor for composting. The pH should be maintained around neutral throughout the whole composting process in order to retain activities of microorganisms. Heap size varies greatly from different compost settings. However, a too small heap size usually leads to loss of heat easily and result in inadequate heating, however too large heap size may result in non homogenized temperature elevation throughout the heap.

Pathogen Survival during Composting

Studies have reported that pathogens are usually eliminated if composting is handled properly according to EPA and USDA guidelines (Ceustermans et al., 2007; Shepherd et al., 2007; Lung et al., 2001; Hess et al., 2004). Ceustermans et al. (2007) reported that

when the temperature and moisture content of the biowaste and garden waste compost heap were 60 ℃ and 60-65%, respectively, *S.* Senftenberg W775 was inactivated within 10 h of composting. Shepherd et al. (2007) revealed that *E. coli* O157: H7 could be inactivated within 14 days of composting in the center of the heap in a field trial when the inoculation level was 7 log CFU g^{-1}. Lung et al., (2001) showed that when the temperature of compost heap reached 45 ℃, *E. coli* O157: H7 and *Salmonella* spp. population declined and became undetectable within 3 day and 2 days, respectively. Hess et al. (2004) conducted a laboratory scale study to determine *E. coli* O157: H7 survival in straw and cattle manure-based compost heaps. They found that *E. coli* and *E. coli* O157: H7 were undetectable within 1 day in compost if the temperature was above 50 ℃. Larney et al. (2003) studied the effects of different carbon source on pathogen survival in compost. They reported that approximately 3 logs of coliform and *E. coli* O157: H7 were inactivated during the mesophilic phase, and carbon source didn't have a significant effect on pathogen elimination.

However, due to the complex nature of composting process, the survival of pathogenic bacteria varies and many environmental factors can affect it. Several studies have already confirmed that pathogens can survive in the finished compost up to several months (You et al., 2006).

Time and temperature are the most important factors for the inactivation of pathogens. During composting, temperature is elevated by microbial activities. The elevation of temperature can kill the pathogens. The major factor for pathogen inactivation is the elevation of the temperature during composting. However, some

composting processes may have extended mesophilic phase or may not reach the thermophilic phase at all. Hess et al. (2004) reported that *E. coli* O157: H7 can survive for over 300 days if the temperature was below 50 ℃. Shepherd, et al. (2007) conducted a composting field trial using dairy manure, old hay, feed waste, a mixture of sawdust and calf feces, and fresh hay. They reported that at both inoculation levels of 7 log CFU g^{-1} and 5 log CFU g^{-1}, *E. coli* O157: H7 survived at the heap's surface for up to 4 months due to different temperature in the heap center and surface. Based on a survey of 9 individual composting heaps on 5 separate poultry farms in South Carolina, Shepherd et al. (2010) reported that *Salmonella* was mostly on the compost surfaces.

Compost indigenous microflora is another factor that affects pathogen survival during composting. The indigenous microflora may serve as a competitive microorganism to the pathogens, thus, can suppress the pathogen survival. Paniel et al. (2010) inoculated pathogenic and indicator microorganisms into compost during different stages, and growth of the inoculated strains were observed during incubation in the autoclaved composts of 10, 12 and 14 weeks. The DGGE analysis demonstrated an increase of microbial diversity up to the cooling phase, and the indigenous microflora appeared to play a significant role in suppressing pathogens. Pathogens survived for over 90 days in autoclaved 12 and 14-week-old compost while they survived for less than 30 days in unautoclaved compost. Ceustermans et al. (2006) also reported that lower levels of indigenous microflora were associated with an increase in the survival time of *S.* Senftenberg W755 in biowaste composting.

C:N ratio is also a factor influencing pathogen survival during composting. Erickson et al. (2009) found that C:N ratio is not a key factor for *L. monocytogenes* survival in compost while *E. coli* O157: H7 is able to survive significantly longer in 40: 1 C:N ratio heap than in 30: 1 or 20: 1 compost heaps.

For poultry waste composting, ammonia volatilization is an important factor contributing to the elimination of pathogens and compost quality. Singh et al. (2012) simulated early phase of poultry waste based composting process on fresh poultry compost in lab, and reported *Salmonella* reduction was faster during the come-up time due to higher ammonia volatilization during thermal exposure.

Recontamination and Regrowth of Pathogens in Finished Compost

The finished compost normally contains low levels of bacteria while survived from the thermophilic phase; however, it is possible that pathogens can recontaminate or grow in the compost during cooling phase. Studies reported pathogens reintroduced or survived in finished compost have the ability to regrow to high levels in processed poultry and dairy-based manure under favorable conditions (Kim et al., 2009, 2010; Zaleski et al. 2005; Pietronave et al., 2004). However, the regrowth potential is affected by many factors.

Indigenous microflora in the finished compost is considered to be an important factor which significantly affects the regrowth of pathogens. Sidhu et al. (2001) monitored the regrowth of *Salmonella* in composted biosolids. In non-sterilized compost, *Salmonella* can only grow to a maximum population density of less than 10^3 CFU g^{-1}, as compared

with 10^8 CFU g^{-1} in autoclaved biosolids, suggesting the indigenous microflora significantly reduced the regrowth potential. The regrowth potential of *E. coli* O157: H7, *Salmonella* and *L. monocytogenes* in dairy waste based compost was investigated by Kim, et al. (2009). Water extract of commercially available dairy compost was used as a model system. Their results also demonstrated that there is efficient nutrient for pathogen regrowth in dairy-based compost, but indigenous microflora may suppress the regrowth. Kim et al. (2009) studied pathogen regrowth by taking consideration of variables such as moisture content, bacteria species, background microflora, different stages of composting and acclimation at room temperature in finished compost. The potential of regrowth for all pathogens were ca. 0.7-1.4 and ca. 4-6 log CFU g^{-1} in unautoclaved and autoclaved finished compost, respectively. Their results revealed that indigenous microflora were a major factor affecting pathogen growth. To validate laboratory observations, Kim et al. (2010) studied the growth and survival of pathogens in compost under greenhouse setting when background microflora level is low. At the initial population of ca. 1 log CFU g^{-1}, there were ca. 1-3 log CFU g^{-1} increase of *Salmonella* and *E. coli* O157: H7 in autoclaved finished compost samples with different moisture contents. However, no pathogen growth was observed in nonautoclaved compost samples. Further research about the indigenous microflora effects on the pathogen growth was conducted. A Mixture of 3 strains of *E. coli* was inoculated into finished compost to yield a ca. 2 log CFU g^{-1} concentration. The growth potential of pathogen was 1.42 to 5.60 log CFU g^{-1} in autoclaved compost, while nonautoclaved compost did not support pathogen growth.

Their results suggested that the level of indigenous microflora is important factor to the regrowth in compost (Kim, et al., 2011).

Moisture content in the finished compost is considered as a key factor for the regrowth of pathogens because bacteria need a high water activity (a_w) environment to metabolize and replicate. The effect of moisture content on pathogen growth was validated by the greenhouse study (Kim et al., 2010). The *Salmonella*, *E. coli* O157 and *L.* monocytogenes populations increased by 2.1-3.9 log CFU g^{-1} with the initial moisture content of at least 40%, as compared with ca. 1 log CFU g^{-1} increase with the pathogen with 30% moisture content. The results suggested that higher moisture content provides enough water for the growth of pathogens under favorable conditions. Zaleski et al. (2005) reported the regrowth of fecal coliform and slightly increase of *Salmonella* in biosolids in a concrete bed after rainfall events, suggesting the water accumulation promoted regrowth of fecal coliform but the increase of *Salmonella* may result from reintroduction by animals. Pietronave et al. (2004) showed that besides the significant influence of indigenous microorganisms, *Salmonella* and *E. coli* grew in compost with a moisture content between 40% and 80% but not at 10%, indicating that the growth of pathogens requires higher moisture content in compost. Russ et al. (1981) studied the regrowth potential of *Salmonella* spp. in composted sludge. Their results showed that the growth occurred in the moisture content of over 20%.

The influence of composting temperature to the regrowth potential of pathogens was investigated by Hess et al. (2004). There was a 1 and 2 log increase of *E. coli* O157:H7 when the composting temperature was maintained at >50 ℃ for 2 days and 40 ℃ to 50 ℃

for 1 day, respectively. Storage temperature is also critical to the regrowth of pathogens in finished compost. In the greenhouse study, Kim et al. (2010) revealed that pathogens grew to higher levels in autoclaved dairy compost during warm seasons, such as the spring and summer months, as compared with cold months. Furthermore, compost maturity is also a factor to pathogen regrowth. Sidhu et al. (2001) suggested that there is a negative correlation (R^2=-0.85) between the maturity of compost and the *Salmonella* inactivation rate in non-sterilized finished compost. The decline of growth potential was most likely resulted from the nutrient limitation in the finished compost. However, they suggested that the influence of compost maturity is not significant as compared to indigenous microflora.

Bioaerosols and Health Risks

Some inadequately processed compost may still contain low levels of pathogens (Shepherd, et al., 2007; Erickson et al., 2009). Also, the compost heap can get recolonized with pathogens from the environment. Those pathogens in finished compost have the potential to regrow to high level under certain conditions (Zaleski et al., 2005; Kim, et al., 2009, 2010). The temperatures on compost heap surfaces during thermophilic phase are usually below the lethal temperatures reached inside the heaps. Previous studies have demonstrated that the compost surface is the area which is considered having high risk of pathogen survival and regrowth (Shepherd et al., 2007, 2009). Thus, the control of pathogens on the surface of finished compost heaps is essential for preventing the spread of pathogens into the environment and food crops.

Composting facilities can create aerosols because of the small size of compost particles. Aerosols have been strongly linked to the spread of foodborne pathogens to fresh produce (Viau et al., 2008), and also cause occupational health problems (Poulson et al., 1995; Douwes et al., 2000). Bioaerosol is defined as aerosolized biological particles (Cox et al., 1995). According to Dowd et al. (2000), the size of bioaerosol can vary from 0.02 to 100 μm. Importantly, bioaerosols can be transferred from soil and compost surfaces into the air which potentially can be a transmission pathway for human pathogens. Lighthart et al. (1995) suggested that these particles can serve as a raft for pathogen. However, a lack of standardized bioaerosol sampling protocols limits the knowledge of aerosol transportation and risks (Pillai et al., 2011).

Bioaerosls are considered as potential contamination sources and the cause of occupational disease. The risks of bioaerosols associated with composting and agricultural activities has been studied. Le Goff et al. (2009) used 16S rDNA and 18S rDNA techniques to investigate the aerosols produced during the thermophilic phase of composting. Species of *Thermomyces, Aspergillus, Penicillium, Geobacillus, Planifilum, Thermoactinomyces, Saccharopolyspora, Thermobifida* and *Saccharomonospora* dominated the population of bioaerosols. They also pointed out that these microorganisms may represent potential indicators of composting bioaerosols in air. Purdy et al. (2009) quantified and sized ambient aerosolized dust collected in and around 4 dairy facilities. Particulate matter (PM_{10} and $PM_{2.5}$) was used as the index to study. A higher mean concentration of dust in the winter was observed (PM10 = 97.4 ± 4.4 μg m^{-3}; PM2.5 = 32.6 ± 2.6 μg m^{-3}) compared with in the summer (PM10 = 71.9 ± 5.0 μg m^{-3};

PM2.5 = 18.1 ± 1.2 μg m^{-3}). They found that the aerosol concentration in dairy facilities exceeded national air quality standards in winter. Fischer et al. (2008) conducted a 3 year study of composting facilities about emission and dispersal of microorganisms. They revealed that airborne microorganisms reached highest levels during compost turning, e.g. 2.4×10^6 CFU /m^3 for thermophilic actinomycetes and about 10^5 CFU /m^3 for other microorganisms. Poulsen et al. (1995) reported that aerosolized gram-negative bacteria can reach 10^6 CFU /m^3 depending on composting process. They also suggested that problems of average versus peak airborne exposure, total versus inhalable aerosol exposure, microbial variability, viable versus total microorganisms and static area air sampling versus personal air sampling should be studied. A study focused on microbial diversity of aerosols produced during compost screening procedure was conducted by Bru-Adan et al. (2009). There was a great diversity of bacteria and fungi in aerosols near the screening area; however, percentage of bacteria cultivability was low in aerosols. Tendal et al. (2010) conducted a study on aerosols in a field of strawberries. Due to the handling of organic materials, berry pickers were potentially subjected to higher levels of fungal spores, *Cladosporium* sp., hyphal fragments, pollen, and β-glucan. Another study was conducted in a commercial poultry house to determine the level of antibiotic resistant bacteria in biological aerosols. Brooks et al. (2010) found the concentration of antibiotic resistant bacteria in aerosol in the house was higher (4×10^6 CFU g^{-1}) compared to the concentration out of the house (6.7×10^3 CFU g^{-1}). *Staphylococcus* bacteria accounted for at least 90% of cultured aerobic bacteria. Three Italian composting plants were monitored to evaluate the degree of bacterial contamination. All three plants were the source of

bacteria in aerosols, and *E. coli*, *S. aureus* and *C. perfringens* were found, suggesting that indoor activities were of greatest potential risk (Fracchia et al., 2006).

Although bioaerosols are widely considered as a contaminant source near organic fertilizer facilities, some other studies have controversial results about the public and occupational health risks. Ravva et al. (2011) studied bioaerosols of compost heaps in two dairy farms in California using 16s RNA sequencing. The aerosols were collected 2 to 3 m away from the heaps, and subjected to sequencing analysis. They indentified prevalent strains in aerosols and compost heaps and suggested that the aerosol sequences may not originate from manure. Another study was focused on the dispersion of pathogenic bioaerosols generated by slurry spreading. Aerosols containing *E. coli* were not detectable from a distance of 250 m. When using the contaminated water for irrigation, pathogens were not detectable from the phylloplane one month after irrigation. Their results concluded that the public health risk is low when the slurry was applied by a rain gun sprayer (Hutchison et al., 2008). Breza-Boruta et al. (2010) found that total fungi, *Salmonella*, *E. coli*, actinomycetes and *Staphylococci* were predominant in air from compost pile storage places, but no significant emission of bacterial aerosol was from the aeration chambers. A one year indoor bioaerosol monitoring was conducted in 3 composting facility environments by Coccia et al. (2010). They assessed different sampling methods for collecting bioaerosol samples, and also concluded that in all environments, pathogen contaminants were present but the occupational risk seemed fairly low.

Transportation of Bioaerosols

The motional characteristics of bioaerosol are affected by gravity, electrical forces, thermal gradients, electromagnetic radiation, turbulent diffusion, inertial forces, oxygen concentrations, and relative humidity (Pillai et al., 2002). Also, size, density and shape have influence on how these factors can affect the movement of bioaerosol. For instance, Brownian motion of bioaerosols increases with the decreasing of particle size (Pillai et al., 2002). As particle size increases, the gravitational settling increases, resulting in decrease of Brownian motion (Cox et al., 1995). And this action is more notable with larger particles.

Particle size is a key factor to the transmission of particles. Aerosolized pathogen transmission can be classified into 2 categories: droplet or airborne (Gralton et al., 2011). Droplet is mainly defined as particles that are able to settle down within 1 m area from the contamination source. Airborne are defined as particles that can stay suspended in the air and transmit to longer distances. The World Health Organization (WHO) sets up the standard for the respiratory diseases transmission: droplet transmission for particle size exceeding 5 μm and airborne transmission for particle size less than 5 μm. The droplet transmission (large particles) is mainly decided by the location of contamination source. For example, fresh produce fields adjacent to composting sites are easily contaminated. For airborne transmission (small particles), it depends on environmental factor such as relative humidity and air flow (Gralton et al., 2011), suggesting that the area downwind of compost facilities are more likely to be contaminated with aerosolized pathogens.

Other environmental factors are also critical to the transmission of bioaerosols. Bioaerosols tend to move from high concentration regions such as composting facilities to low concentration regions such as produce fields and municipal buildings. Also, they tend to move from warm regions to cold regions. Other important environmental factors for bioaerosol motion are relative humidity and moisture content. High humidity environment helps bacteria to disperse into air. The transport of bioaerosols can be defined in terms of distance and time. Models have been developed to describe and predict the transportation of bioaerosols in different situations (Dowd et al., 2000). Lighthart et al. (1987) used virus as a sample to set up a model of airborne microbial survival with certain environmental variables. A modified Gaussian plume model including microbial source strength, particles size and local mean weather data was used. The multiple regression illustrates the potential importance of wind as a dilution and survival factor, which means the microbes are diluted by a factor of 10,000 in the first 30 m downwind from the source.

In summary, the evidence suggests that the aerosol transmission has been underestimated in the past. More studies are needed to determine the risk of foodborne pathogen transmission from compost sites to the environment.

Fluorescence and Confocal Microscopy

A fluorescence microscope is basically an optical microscope. Besides the white field observation, it uses fluorescence and phosphorescence to study and observe samples. Under a fluorescence microscope, a sample is illuminated with the light of a

wavelength which excites fluorescence. The illumination/excitation light usually has a specific wavelength which can be recognized and absorbed by the fluorophores. This leads to the emission of the light which has a longer wavelength and lower energy. There are two light filters which ensure that the illumination light is at a specific wavelength and the emission light noise is removed.

A basic fluorescence microscope contains a light source (usually a mercury lamp), the excitation filter, the dichroic mirror, the objective, the emission filter and the detector (fig 1.1).

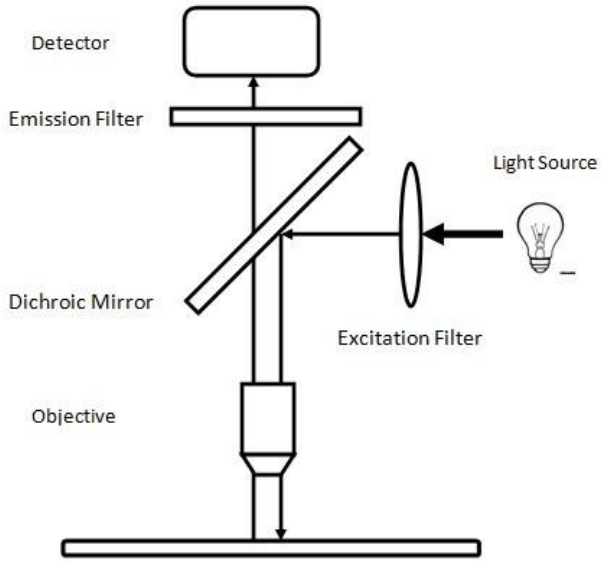

Fig 1.1 basic structure of fluorescence microscope

A confocol microscope is a fluorescent microscope which can increase optical resolution and contrast by using point illumination and a spatial pinhole. These are able to obtain a 3D structural image of the specimen with the elimination of noise light. Where as the whole sample is exposed to the excitation light evenly in conventional fluorescence

microscope, confocal microscope uses point illumination and a pinhole in front of the detector to eliminate out-of-focus signal. Confocal microscope increases the resolution in great level but it decreases the signal intensity in sacrifice.

Studies using confocal microscopy to observe microorganisms in soil samples have been conducted. Sol é et al. (2007) developed a new automated method based on the method of optical series of cyanobacterial to determine its biomass in microbial mats by confocal laser scanning microscopy image analysis (CLSM-IA). The excitation light wavelength was set as 568 nm and images showed the natural autofluorescence emitted by cyanobacteria. Samples were transferred into small plastic tubes and fixed in 2.5% (v/v) glutaraldehyde Sorensen phosphate buffer (0.2 M, pH=7) for 3 h. Then, the samples were washed and stored at 4 ℃ until observed. The smallest unit of a three-dimensional digital image was equivalent to 1.183×10^3 mgC/cm^3 of sediment due to the limitation of resolution.

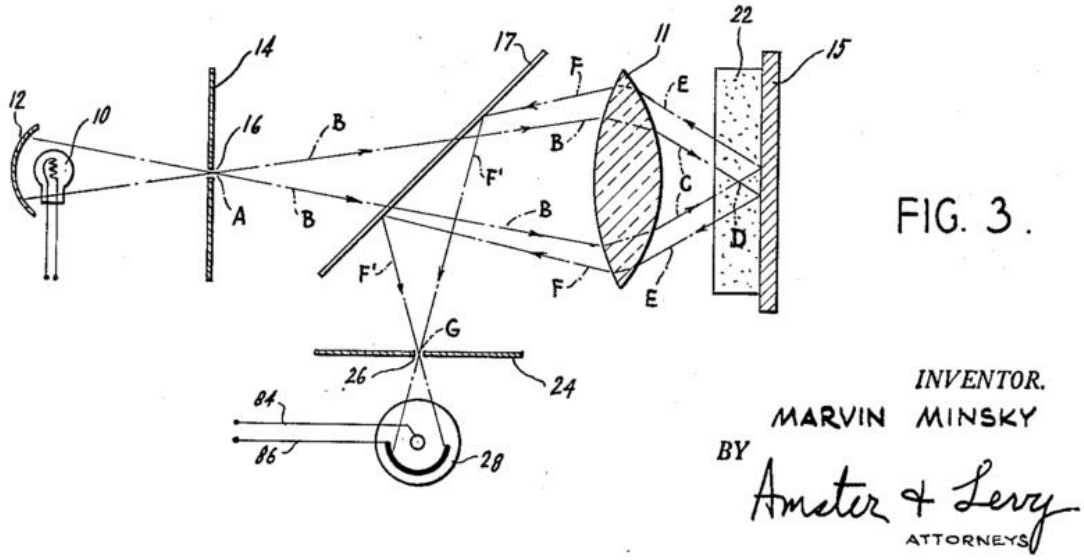

Fig 1.2 Optical path of a reflection confocal microscope, as patented by Marvin Minsky in 1957.

Microscope Observation of Microorganisms in Soil Matrix

Recently, molecular sequencing has been widely used for the investigation of microorganisms in soil or compost materials. But the visual confirmation method is still not well-established. Fluorescence microscopy and confocal microscopy are showing promising potential as a valid method to observe and analyze microorganisms. Due to the complex and heterogeneous nature of soil and compost, observation of microorganisms under these microscopes has major obstacles to overcome.

Multiple labeling fluorescence techniques are powerful ways to achieve simultaneous identification of multiple molecular or structural components in living cells (Kho et al., 2008; Medina et al., 2002). Fluorescent protein constructs have been used as a tool to analyze protein location and interaction within the cell structure (Anderson et al.,

2006). Green fluorescent protein (GFP) is widely used in live cell imaging now. The GFP was cloned from a jellyfish and used for labeling structures within cells and tissues (Charlfie et al., 1994). BFP, CFP, YFP, dsRed, and other different variants of FPs are developed after GFP, however, they are limited by a variety of shortcomings (Remington et al., 2002).

The major drawback of the method of multichannel detection when working with living cells is that the broad (ca. 80 nm) and overlapping emission spectra makes it necessary to sacrifice the signal intensity to separate signals from different fluorophores (Carlsson et al., 1997). Usually, the emission fluorescence needs to be discarded in a great amount to achieve reliable spectral separation. Thus, conventional filter-based spectral-separation technique has a limitation of detecting only 4 fluorophores detection simultaneously (Kho et al., 2008). Inorganic fluorescent probes such as quantum dots are solution to the limitations of conventional organic fluorophores. The Qdots are produced strictly uniform of their size, and the emission light wavelength are associated with their specific sizes. Thus, the emisson spectra of quantum dots conjugates are narrow and symmetric (Michalet et al., 2005). The Qdots are much brighter and photostable. Furthermore, fluorescence lifetime imaging has been used to overcome this problem. Several images need to be taken to determine the lifetime and relative contribution of each FP (Pepperkok et al., 1999). However, all of these methods have their drawbacks.

Spectral imaging and following linear unmixing is known as a processing technique that has been designed for separating overlapping fluorescence signals from fluorescence proteins (Zimmermann et al., 2003). It can be used under whitefield as well

as under confocal mode. It is used when careful selection of excitation sources and emission filters fail to process the samples (Anderson et al., 2006). This technique uses the emission spectra curve data from individual signals to mathematically dicomposition the mixed sample emission spectra (Zimmermann et al., 2005; Berg et al., 2004). Thus, the relative contribution of each signal needs to be available as the spectral profile. The major advantages of this application are that increasing signals can be detected at one time and the data can be gained in a short time without any additional sample treatment. This application can be exploited to image bacteria in different matrix (Wolf et al., 2005).

However, the linear unmixing technique is limited by factors such as the image background level, noise, and the relationship of the emission peaks to the detection channels (Anderson et al., 2006) when imaging living specimens. A maximum of 7 fluorophores can be detected simultaneously by this technique (Tsurui et al., 2000).

Immunofluorescence (IF) microscopy is a technique used for fluorescence microscopy and it is used primarily for living samples. This technique uses the specific affinity of the antibody to the antigens to link the target protein to fluorescence dyes. It is a very sensitive serological test which can use a microscope to visualize the location of a specific protein or cell (Janse et al., 2009). IF uses a primary antibody to recognizes and bind to the target molecule. A secondary antibody then binds to the primary antibody to amplify the fluorescence signal (Welter et al., 2002). Ammar et al. (2005) used IF technique to study distribution of the bacterium corn stunt spiroplasma in vector leafhoppers. The study suggested that the IF technique is effective to label proteins in

tissue matrix. Welter et al. (2002) also verified their IF protocol is suitable for labeling proteins in microorganisms.

Fluorescence in situ hybridization (FISH) uses fluorescence probes to detect and localize the presence or absence of specific DNA sequences on chromosomes. Whiley et al. (2011) successfully detected *Legionell*a spp. in soil samples using Fluorescent in situ Hybridization (FISH) with rRNA targeted oligonucleotide probes. Under the confocal microscope, several methods were tried to minimize the background autofluorescence and non-specific binding, such as the use of a blocking agent, UV light treatment, image subtraction of a nonsense probe and spectral unmixing. They reported that FISH can enhance the sample intensity and sensitivity while spectral unmixing can minimize the influence of autofluorescence. Eickhorst et al. (2008) studied soil microbial diversity using the FISH assay. Soil samples were fixed in 4% paraformaldehyde-PBS solution in 4℃ for 5 h, and then the fixed samples were FISH stained and observed. The main disadvantage of FISH assay in soil is the stained sample is strongly affected by autofluorescence (Bertaux et al., 2007), and the maximum autofluorescence in soil samples are emission light at 534-558 nm wavelength (Eickhorst et al., 2008). Thus, double excitation was used in epifluorescence microscopy to enhance the visual ability in some studies. FISH assay in combination with confocal laser scan microscopy was used to observe ectomycorrhizal fungus Laccaria bicolor S238N in other studies (Bertaux et al., 2005).

Summary

In recent years, fresh produce related foodborne disease outbreaks are drawing more attention than before. Animal waste directly applied to agricultural fields is one of the suspected contamination sources. Composting is recommended by USDA to treat the animal wastes in order to be considered as a safe and nutritious fertilizer. However, it is possible that pathogens can survive, recolonize and regrow on compost heap surfaces to a hazardous level. Furthermore, bioaerosols can be generated on compost surfaces, and subsequently carry pathogens and travel through air to contaminate fresh produce in the nearby field. The ability of pathogens to survive in different sizes of compost particles varies due to some intrinsic and extrinsic factors. However, the survival rate of pathogens in compost particles with different sizes and moisture content has not been studied yet. Additionally, visualization of pathogens associated with compost particles by microscopy may provide more insights about the pathogen survival strategies.

The objectives of this study were as follows:

- Investigate the impacts of particle size and moisture content of sample on pathogen survival rate in finished compost in different seasons under greenhouse conditions;
- Develop a valid method for microscopy analysis of GFP-labeled *E. coli* in compost samples.

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CHAPTER TWO

INFLUENCE OF COMPOST PARTICLE SIZE ON PATHOGEN SURVIVAL UNDER GREENHOUSE CONDITION

Abstract

Animal waste directly applied to agricultural field is one of the possible contamination sources for fresh produce. Although manure-borne pathogens can be eliminated by proper composting process, pathogens are able to survive, recolonize and regrow on compost heap surfaces. Due to the outdoor nature of composting process, bioaerosols with different particle sizes can be generated on compost surfaces which can carry pathogens, travel via air and contaminate fresh produce field nearby. This study was to investigate the survival of *Escherichia coli* O157: H7 and *Salmonella enterica* Typhimurium in dairy compost with different particle sizes as affected by initial moisture content and seasonality under greenhouse conditions. The mixture of avirulent *E. coli* O157: H7 and *S.* Typhimurium strains were inoculated into the finished composts with initial moisture content of 20, 30 and 40%. Then, the finished compost samples were sieved into three portions with particle sizes of >1000, 500-1000 and <500 μm, and stored in greenhouse for 30 days. At selected intervals, compost samples were enumerated for the target pathogens.

The moisture contents in compost samples dropped rapidly to under 10% within 5 days of storage followed by gradual decline till 30 days in all treatments. For compost with moisture contents of 20 and 30%, the average *Salmonella* reductions in compost with particle sizes of >1000, 500-1000 and <500 μm were 2.15, 2.27 and 2.47 log CFU g-

1 within 5 days of storage in summer, respectively, as compared with 1.60, 2.03 and 2.26 log CFU g-1 in late fall, and 2.61, 3.33 and 3.67 log CFU g^{-1} in winter, respectively. For samples with initial moisture content of 40%, the *Salmonella* reductions in compost with particle sizes of >1000 and <1000 μm were 2.14 and 3.17 log CFU g^{-1} within 5 days in summer, respectively, as compared with 3.17 and 3.16 log CFU g^{-1} in late fall, and 2.93 and 3.36 log CFU g^{-1} in winter, respectively. For compost with moisture contents of 20 and 30%, the average *E. coli* O157: H7 reductions in compost with particle sizes of >1000, 500-1000 and <500 μm were 1.98, 2.30 and 2.54 log CFU g^{-1} within 5 days of storage in summer, respectively, as compared with 1.70, 2.56 and 2.90 log CFU g^{-1} in winter, respectively. For samples with initial moisture content of 40%, the *E. coli* O157: H7 reductions in compost with particle sizes of >1000 and <1000 μm were 2.08 and 2.48 log CFU g^{-1} within 5 days in summer, respectively, as compared with 2.20 and 2.84 log CFU g^{-1} in winter, respectively.

Our results revealed that both *E. coli* O157: H7 and *Salmonella* in compost with larger particle size survived better ($P<0.05$) than the compost with smaller particle size, and the initial rapid moisture loss in compost may contribute to fast inactivation of pathogens in the finished compost. For the same season, the pathogens in the compost with the same particle size survived better ($P<0.05$) with initial moisture content of 20% than 40%.

Introduction

According to the Centers for Disease Control and Prevention (CDC), there were a total of 48 million foodborne illnesses, 128,000 hospitalizations and 3,000 deaths associated with food consumption in the United States each year (CDC, 2011). The number of foodborne pathogen outbreaks related to fresh produce has increased significantly in recent decades (CAST, 2009). From 1973 through 1997, a total of 190 outbreaks, 16,058 cases, 598 hospitalizations, and 8 deaths were reported to relate to fresh produce (Sivapalasingam et al., 2004). However, from 1990 through 2003, there were 554 outbreaks and 28,315 cases related to fresh produce (Dewaal et al., 2006). These outbreaks are mostly caused by some well-known foodborne pathogens such as *Escherichia col*i O157: H7, *Salmonella* spp., and *Listeria monocytogenes*.

The mechanism of pathogen transmission to fresh produce remains unclear. The possible transmission routes of pathogens to fresh produce include manure, composts, manure-amended soil, wild animals and irrigation water (Doyle et al., 2008). Organic fertilizers including animal manure are widely used in agricultural production in the United States, with manure spreading accounting for 5% of total U.S. cropland (MacDonald et al., 2009). However, manure is not considered as a safe source of nutrients for fresh produce production since the pathogens can survive for several months in manure-amended soil (Islam et al., 2005; Jiang et al., 2002).

Composting is one of the recommended means for waste treatments to eliminate or reduce pathogens in manure on farms. In the U.S., animal husbandry rapidly changes from small, isolated home-raising to large-scaled, concentrated animal raising facilities,

thus, more animal wastes are generated and composted than before. Previous studies provided evidences that pathogens were either reduced or eliminated during the composting process (Lung et al., 2001; Ceustermans et al., 2007). But there are still possibilities for pathogens to survive during composting because of heterogeneity of compost heaps (Shepherd et al., 2010). Pathogen survival during composting varies largely among different seasons (Erickson et al., 2010), it is also affected by time, temperature, type of manure, moisture content and other factors (Shepherd, et al., 2007; Droffner and Brinton, 1995). Additionally, studies reported that pathogens reintroduced or survived in finished compost have the ability to regrow to high levels in dairy manure-based compost under favorable conditions (Kim et al., 2010). Therefore, the contaminated compost can play an important role in the presence of foodborne pathogens on fresh produce (Oliveira et al., 2011). Since pathogens on fresh produce are able to survive from the farm to the fork (Tomas-Callejas et al., 2011), it is necessary to investigate how the foodborne pathogens in manure or compost are transmitted to fresh produce during preharvest.

Studies have demonstrated that foodborne pathogens survived well on compost surface. Shepherd et al. (2009) investigated 9 individual composting heaps on 5 separate poultry farms in South Carolina. In the survey, there were ca. 94 and 76% of surface samples positive for coliforms and presumptive *Salmonella* spp., respectively, as compared to less than 50 and 26% in the internal samples. Their results on the microbiological quality of all the compost heaps revealed that pathogens can survive at compost surfaces during composting process. Wichuk et al. (2011) conducted a field

study using 3 strains of fungi to inoculate compost heaps at various locations. Survival of fungal pathogens was observed near the surfaces. Shepherd et al. (2007) conducted a dairy manure-based composting field trial, and reported that *E. coli* O157: H7 at both inoculation levels of 10^7 CFU g^{-1} and 10^5 CFU g^{-1} survived at the heap's surfaces for up to 4 months.

Finished compost particles on the composting surface may travel through air as bioaerosols from composting site to vegetable production site nearby. Manure-borne pathogens may become airborne by adhering to compost particles (Coccia et al., 2010), and this provides one of the routes for pathogen transmission. Bioaerosols are composed of inert particles and the attached microorganisms (Le Goff et al., 2009), and they vary greatly in size (Dowd et al., 2000). There are a few studies on bioaerosol generation, transportation and pathogen contamination associated with composting operation. Purdy et al. (2010) quantified and sized ambient aerosolized dust around 4 dairy facilities of New Mexico and found the mean concentration of dust in the winter was significantly higher than it in the summer, with the highest concentration as above 100 μg m^{-3}. Fisher et al. (2007) conducted a three year study at 9 different composting facilities in Germany and found that thermophilic actinomycetes population was as high as 2.4×10^6 CFU m^{-3} in drain flow situation while levels of other groups of microorganisms were around 10^5 CFU m^{-3}. Coccia et al. (2010) conduct a year-long indoor bioaerosol monitoring experiment in three working environments of a municipal composting facility and found that the occupational risk seems fairly low. Ravva et al. (2011) collected aerosols at two dairies and analyzed by 16sDNA sequencing. Their results showed that the aerosol

sequences may not originate from manure. Overall, aerosols generated from composting heaps have been suspected to transport foodborne pathogens to fresh produce, but how the pathogens survive in aerosol in the open environment has not been studied yet.

The purpose of this study was to investigate the survival of *E. coli* O157: H7 and *Salmonella* spp. in compost on composting surface as affected by compost particle size, initial moisture content and seasonality under greenhouse conditions.

Material and Methods

Preparation of finished compost. The commercial Black Kow dairy compost (Black Gold Compost Company, Oxford, FL, USA) was used in this study. It was purchased from a local Lowe's store and kept at room temperature. As stated on the label, this product has 0.5% total nitrogen, 0.5% available phosphate (P_2O_2), 0.5% soluble potash (K_2O), and no more than 1% chlorine. Finished compost sample was dried under a hood until the moisture content was decreased to less than 20%. This dried sample was then screened by a sieve (sieve pore size, 3 by 3 mm) to a size of less than 3 mm. Initial moisture content of the finished compost sample was measured with a moisture analyzer (Model IR-35 Infrared analyzer; Denver Instrument, Denver, CO). The pH of the compost sample was determined by adding 1 g sample in 50 ml distilled water, then stirred for 2 min, and measured by a pH meter (Orion Star 2-star meter, Thermo Scientific, Beverly, MA).

Bacterial cultures. Avirulent, ampicilin-resistant and green fluorescent protein (GFP)-labeled *E. coli* O157: H7 strain B6914 (kindly provided by Dr. Pina Fratamico at

the United States Department of Agriculture, Agricultural Research Service – Eastern Regional Research Center) and avirulent *Salmonella* Typhimurium strain 8243 (kindly provided by Dr. Roy Curtis III, Washington University, St. Louis, MO) were used. *S.* Typhimurium strain was induced to be resistant to 100 μg of rifampin ml^{-1} (Fisher Scientific, Fair Lawn, NJ) using the gradient plate method (Rice et al., 2005). Bacterial cultures were stored at -80 ℃ in tryptic soy broth (TSB; Becton Dickinson, Sparks, MD) with 20% glycerol.

Inoculum preparation. The frozen stock cultures of *S.* Typhimurium and *E. coli* O157: H7 were thawed and streaked on tryptic soy agar (TSA; Becton Dickinson, Sparks, MD) supplemented with 100 μg/ml of rifampin and ampicilin, respectively, and grown at 37 ℃ for 24 h (Kim et al., 2008). Single colonies of *S.* Typhimurium and *E. coli* O157: H7 on TSA supplemented with rifampin (TSA-R) or ampicilin (TSA-A) were transferred into 50 ml of TSB supplemented with rifampin (TSB-R) or ampicilin (TSB-A), respectively, and incubated in a shaking incubator (100 rpm, 37 ℃) for 24 h. Each overnight culture was centrifuged at 8,000 rpm for 10 min, washed 3 times with sterile saline (0.85% NaCl). The optical density at 600 nm (OD_{600}) of each culture was adjusted to 0.7 with sterile saline to achieve a bacterial population of ca. 10^9 CFU ml^{-1}. Each culture was enumerated by plating proper dilutions of the inoculum in duplicate on TSA-A or TSA-R plates.

Sample Inoculation and placement. The moisture content of above finished compost sample was adjusted to 20, 30 or 40% with sterile tap water as needed. A 1,000 g portion compost sample of different moisture content was weighted in a sterile tray. *E.*

coli O157: H7 and *Salmonella* inocula were inoculated into the finished compost samples at a ratio of 1: 100 (vol: wt) with a sterile spray nozzle (sterilized with 70% ethanol and rinsed with sterile tap water) to yield a final concentration of ca. 10^7 CFU g^{-1}. The inoculated compost samples were continually mixed by hands wearing sterile gloves (Sterile nitrile gloves, Kimberly-Clark, Neenah, WI) for 15 min under a biological hood. The above compost samples with 20 and 30% moisture content were then sieved into 3 portions: >1000, 500-1000 and <500 μm using standard sieves (#18 and #35, VWR). Whereas, compost sample with 40% moisture content was separated into >1000 and <1000 μm portions due to large particle size formed in compost with high moisture content. From each size portion, a 60 g sample was placed into a plastic tray (4×4 cm) at a depth of 3-4 cm. Duplicate samples from each moisture content and particle size at each sampling point were prepared and sampled throughout the trial. The trays were put into a plastic transparent container (50×140 cm) and stored in greenhouse up to one month without further adjustment of moisture content. The experiment was conducted during Nov.-Dec., 2011, Jan.-Feb., 2012 and Jul.-Aug., 2012 for fall, winter and summer trials, respectively. For each season, the second trial was initiated one week after the beginning of the first trial.

Environmental conditions of a greenhouse. Temperature and relative humidity (RH) in the experimental unit in the greenhouse were measured and recorded by the remote monitoring system (Argus control system Ltd, White Rock, BC, Canada). Greenhouse room was set to have an upper limit of 30.5 ℃ and 700W m^{-2} for temperature and light intensity, respectively. Due to the fact that the greenhouse is designed for plant

growth, a low temperature control limit was set at 16 ℃. Forced ventilation and evaporated cooling were used to control temperature when its too high and occasionally shading was used to control light intensity. Considering the average temperature outside was lower than 16 ℃ in the winter and higher than 30.5 ℃ in the summer, temperatures in these trials were not as extreme as actual temperatures outside.

Microbiological analysis. Samples were taken and analyzed on days 0, 1, 3, 5, 10, 15, 20 and 30 during storage. A 5 g sample from each plastic tray was taken using sterile tongue depressor, mixed with 45 ml of universal pre-enrichment broth (UPB; Becton Dickinson) in a Whirl-pak stand-up bag (Nasco, Fort Atkinson, WI) and then shaken vigorously for 1 min. The sample was enumerated by serially diluting in saline and plating on TSA-A and XLT-4-R in duplicate using an Autoplate® 4000 spiral plater (Spiral Biotech Inc., Bethesda, Md.) for *E. coli* O157: H7 and *Salmonella*, respectively. The plates were incubated at 37 ℃ for 24 h and 48 h for TSA-A and XLT-4, respectively.

When the direct plating method failed to detect any colony (detection limit of 1.30 log CFU g-1), the samples were enriched in UPB at 37 ℃ for 24 h, followed by selective enrichment in TSB-A or Rappaport-Vassiliadis broth with rifampin (RV-R) at 37 ℃ for *E. coli* O157: H7 and *Salmonella*, respectively. After incubation for 24 h, a loopful of secondary enrichment culture was streaked onto TSA-A and XLT4-R for *E. coli* O157: H7 and *Salmonella*, respectively. Selected colonies grown on each selective media were confirmed by *E. coli* and *Salmonella* latex agglutination tests, respectively (Oxoid Inc., Basingstoke, Hampshire, UK).

Statistical analysis. For each season, there were 2 separate trials and in each trial, experiments were conducted in duplicate. Total bacterial counts were converted to log CFU g-1 on dry weight basis and log reductions of each pathogen within 5 initial days and after 30 days in each trial were subjected to analysis of variance (ANOVA), followed by the least significant different (LSD) analysis with Statistical Analysis System (SAS, version 9.1; SAS Institute Inc, Cary, NC). A P-value less than 0.05 was considered significant different among different treatments. In this study, three factors being examined were season (Summer, late Fall and Winter), moisture content (20, 30 and 40%), and particle size (>1000, 500-1000, and <500 μm). Also, the correlation between pathogen reduction and dehydration rate within 5 days in different season was analyzed by SAS. A linear regression model was used to predict the change of *E. coli* O157: H7 and *Salmonella* spp. reduction according to the dehydration rate in different seasons.

Results

Three greenhouse trials were performed during Nov.-Dec., 2011 (late fall trial), Jan.-Feb. 2012 (winter trial) and July-Aug 2012 (summer trial). The average values of temperature in greenhouse were 21.3, 21.7 and 28.4℃ for late fall, winter and summer trials, respectively, while the average values of relative humidity (RH) in greenhouse were 41.3, 36.0 and 64.4% for late fall, winter and summer trials, respectively. Fig 2.1 presented the average temperature, RH and light intensity inside the greenhouse for a typical day.

Initial pH values for compost samples used in late fall, winter and summer trials were 7.93±0.09, 7.66±0.01 and 7.77±0.04, respectively. The levels of background mesophilic microorganisms in these samples ranged from 6 to 8 log CFU g^{-1}. All compost samples for these trials were negative for both *E. coli* O157: H7 and *S.* Typhimurium.

Changes of moisture content in compost stored in different seasons. The experiments were conducted with no further moisture adjustment after the compost samples were placed in greenhouse. The moisture change in each sample during one-month storage is shown in Fig 2.2-2.10.

In general, the moisture contents of compost were reduced from 40, 30 and 20% to less than 10% within 5 days for all samples during late fall and winter trials (Fig 2.2 A, 2.3 A, 2.4 A, 2.5 A, 2.6 A and 2.7 A). After 5 days, moisture content of compost samples stayed at less than 10% until day 30 in these trials. For summer trial, the moisture contents of compost samples with <500 μm particle size reduced from 30 and 20% to less than 10% within 5 days (Fig 2.8 A, Fig 2.9 A and Fig 2.10 A), Whereas, for compost samples with particle size of >1,000 μm, the moisture content increased ca. 5% within one day, followed by a rapid decline, and then stayed at 5-25% from day 5 to 30 (Fig 2.8 A, Fig 2.9 A and Fig 2.10 A).

For the same initial moisture level, moisture content of compost samples with larger particles decreased slower than those with smaller particle sizes (Table 2.1). As an example, in samples with >1000 μm size and 30% initial moisture content in late fall trial, it had a 4.02%/d dehydration rate on average within the first 5 days, as compared to

5.26 and 5.50%/d in compost samples with sizes of 500-1000 and <500 μm, respectively. For samples with 30% initial moisture content in winter trial, there were 4.48, 4.99 and 4.90%/d dehydration rate of samples with >1000, 500-1000 and <500 μm particle sizes, respectively. Moreover, in samples with 30% initial moisture content in summer trial, moisture content in the >1000, 500-1000 and <500 μm samples had dehydration rates of 1.69, 3.82 and 4.70%/d within 5 days, respectively.

Statistical analysis indicated that dehydration rate within initial 5 days in compost samples with particle size of >1000 μm was significant lower ($P<0.05$) than samples with particle sizes of 500-1000 and <500 μm in samples with all moisture levels in summer trial and samples with moisture content of 30% in late fall trial. However, the difference of sample dehydration rates was not statistically significant among particle sizes of compost samples in winter trial.

Survival of *S.* Typhimurium and *E. coli* O157: H7 in the finished compost. The initial inoculation levels for *S.* Typhimurium and *E. coli* O157: H7 were at 6.5-7.5 log CFU g-1. Under greenhouse conditions, both *S.* Typhimurium and *E. coli* O157: H7 populations declined rapidly within 5 days and then followed by gradual reduction till the last sampling day (day 30). Thus, the log reduction in the first 5 days was used to analyze the inactivation rates of both pathogens.

Table 2.1 summarized the pathogen reduction and dehydration rate in compost samples during initial 5 days of storage for 3 seasons as effected by compost particle size and initial moisture content.

S. Typhimurium**:** For late fall trial, the population of *Salmonella* decreased by 3.19 and 3.16 log CFU g^{-1} within 5 days for the compost samples (40% initial MC) with particle sizes of >1000 and <1000 μm, respectively (Fig 2.2 B, Table 2.1). *Salmonella* population decreased by 1.80, 2.35 and 2.74 log CFU g^{-1} within 5 days for the compost samples (30% initial MC) with particle sizes of >1000, 500-1000 and <500 μm, respectively (Fig 2.3 B, Table 2.1), as compared to pathogen reduction of 1.39, 1.70 and 1.78 log CFU g^{-1} for 20% initial MC compost samples with particle sizes of >1000, 500-1000 and <500 μm, respectively (Fig 2.4 B, Table 2.1).

For winter trial, *Salmonella* population decreased by 2.93 and 3.36 log CFU g^{-1} within 5 days for compost samples (40% initial MC) with particle sizes of >1000 and <1000 μm, respectively (Fig 2.5 B, Table 2.1). For the samples with 30% initial moisture content, population of *Salmonella* decreased by 2.33, 3.23 and 3.68 log CFU g^{-1} within 5 days for the >1000, 500-1000 and <500 μm samples, respectively (Fig 2.6 B, Table 2.1), as compared to pathogen reductions of 2.88, 3.42 and 3.66 log CFU g^{-1} with compost samples of 20% initial MC, respectively (Fig 2.7 B, Table 2.1).

For summer trial, there was an average *Salmonella* population reduction by 2.14 and 3.17 log CFU g^{-1} within 5 days for the compost sample (40% initial MC) with particle sizes of >1000 and <1000 μm, respectively (Fig 2.8 B, Table 2.1). *Salmonella* population decreased by 2.35, 2.61 and 2.84 log CFU g^{-1} within 5 days for the compost samples (30% initial MC) with particle sizes of >1000, 500-1000 and <500 μm, respectively (Fig 2.9 B, Table 2.1), as compared to 1.95, 1.93 and 2.10 log CFU g^{-1} for the samples with 20% MC, respectively (Fig 2.10 B, Table 2.1).

E. coli O157: H7: Two trials were conducted for *E. coli* O157: H7 in compost with different particle sizes. For winter trial, the population of *E. coli* O157: H7 decreased by 2.20 and 2.84 log CFU g^{-1} within 5 days for the compost samples (40% initial MC) with particle size of >1000 and <1000 μm, respectively (Fig 2.5 C, Table 2.1). As for samples with 30% initial MC, *E. coli* O157: H7 population decreased by 1.28, 2.05 and 2.68 log CFU g^{-1} within 5 days for the compost samples with particle sizes of >1000, 500-1000 and <500 μm, respectively (Fig 2.6 C, Table 2.1), as compared to pathogen reductions of 2.11, 3.07 and 3.11 log CFU g^{-1} for samples with 20% MC, respectively (Fig 2.7 C, Table 2.1).

For summer trial, there was an average *E. coli* O157: H7 population reduction by 2.08 and 2.48 log CFU g^{-1} within 5 days for the compost sample (40% initial MC) with particle sizes of >1000 and <1000 μm, respectively (Fig 2.8 C, Table 2.1). For the samples with 30% initial moisture content, population of *E. coli* O157: H7 decreased by 2.14, 2.40 and 2.54 log CFU g^{-1} within 5 days for the >1000, 500-1000 and <500 μm samples, respectively (Fig 2.9 C, Table 2.1). However, in compost samples with 20% initial MC, *E. coli* O157: H7 decreased by 1.82, 2.19 and 2.53 log CFU g^{-1} for the samples with particle sizes of >1000, 500-1000 and <500 μm, respectively (Fig 2.10 C, Table 2.1).

Statistical analysis for pathogen survival in the finished compost. Three environmental factors being examined were season (late fall, winter and summer), initial moisture content (20, 30 and 40%), and particle size (>1000, 500-1000, and <500 μm).

Within 5 days of storage: For *Salmonella*, there was no difference ($P > 0.05$) in the log reductions as affected by different particle size in late fall and summer trials except for the samples with 40% initial MC in summer. In winter trial, there was no significant difference ($P > 0.05$) in log reductions as affected by different particle size when the initial MC was 20 and 40%. However, the log reduction of compost sample with particle sizes of >1000 and <500 μm were significantly different ($P < 0.05$) when initial MC was 30% (Table 2.1). For the analyses of impact of initial moisture content on *Salmonella* spp. reduction, there was no significant difference between log reductions in winter and summer trials. In late fall trial, *Salmonella* log reduction of 40% MC sample was significantly different from samples with moisture content of 30 and 20% when the particle size was >1000 μm (Table 2.1).

For *E. coli* O157: H7, there was no difference ($P > 0.05$) in the log reductions as affected by different particle size in summer. However, for samples with initial MC of 30%, there was a significant difference ($P < 0.05$) in log reductions among samples with particle size of >1000, 500-100 and <500 μm in winter trial (Table 2.1). For the impact of initial moisture level on *E. coli* O157: H7 log reduction, there were no significant difference in *E. coli* O157: H7 log reduction in summer trial when the particle size was the same. However, in winter trial, *E. coli* O157: H7 log reduction of 30% MC sample was significantly different from samples with moisture content of 40 and 20% when the particle size was >1000 μm (Table 2.1).

Entire storage of 30 days: For *Salmonella*, there was no difference ($P > 0.05$) in the log reductions as affected by different particle size when the initial MC was the same

in summer trial. In late fall trial, there was a significant difference between samples with particle sizes of >1000 and <500 μm with an initial MC of 30%. However, when the log reduction affected by moisture content was compared in late fall trial, the data showed that log reductions of samples with 40% initial MC are significantly different from those samples with 20% initial MC. In winter trial, for samples with initial MC of 20%, there was a significant difference ($P < 0.05$) in log reductions between samples with particle sizes of >1000 and <500 μm (Table 2.2). For the analyses of impact of initial moisture content on *Salmonella* spp. reduction within 30 days, there was no significant difference between log reductions in winter and summer trials. In late fall trial, *Salmonella* log reduction of 40% MC sample was significantly different from samples with moisture content of 20% when the particle size was >1000 μm (Table 2.2).

For *E. coli* O157: H7, there was no difference ($P > 0.05$) in the log reductions as affected by different particle size and initial MC in summer trial. However, in winter trial, there was a significant difference ($P < 0.05$) in log reductions between samples with particle size of >1000 and <500 μm when the initial MC were 20% and 30% (Table 2.2). However, when the particle size of samples was the same, there was no significant different log reduction of *E. coli* O157: H7 with different initial moisture contents except for the particle size of >1000 μm with 30% moisture in winter trial (Table 2.2).

The correlation of the pathogen log reduction and dehydration rate of the compost sample were presented in Table 2.3, along with the index of regression with the dependant variable (pathogen log reduction) and independent variable (dehydration rate). The correlation between *Salmonella* log reduction in late fall, *Salmonella* log reduction in

summer and *E. coli* O157: H7 log reductions in summer were 0.98, 0.90 and 0.79, respectively. However, the correlation between *Salmonella* and *E. coli* O157: H7 log reductions in winter were -0.05 and -0.12, respectively. The R^2 showed that in late fall and summer trial, the relationship of pathogen reduction and dehydration rate can be described as a linear regression model, whereas in winter trial, the change of pathogen log reduction was not linear with the dehydration rate.

Discussion

Previous studies have suggested that pathogens may survive, recolonize or regrow on compost heap surfaces (Kim et al., 2009, 2010; Zaleski et al. 2005; Pietronave et al., 2004). When the surface of compost heaps becomes dry, smaller compost particles may form bioaerosols easily as a result of windy condition or compost turning events. Pathogens associated with compost bioaerosols can be blown away and contaminate nearby fresh produce field. Thus, pathogens on finished compost surfaces should be eliminated. The ability for compost particles to generate bioaerosols and how far they can travel are affected by many factors, such as particle size, moisture level, wind, velocity, relative humidity and temperature (Pillai et al., 2011). However, there are no sufficient scientific studies evaluating the behavior of pathogens in the finished compost of different particle sizes. This study was to investigate the impacts of compost particle size and moisture content of compost sample on pathogen survival in different seasons.

In this study, *S.* Typhimurium and *E. coli* O157: H7 were inoculated into the finished dairy compost. Due to the exposure to dry air and high temperature, rapid loss of

water and lack of nutrients, pathogen population declined rapidly in the compost once stored in greenhouse. However, the pathogen survival curves showed an extensive tailing after 5 days of storage time regardless of season and initial moisture content. This tailing effect of pathogen inactivation was also observed in other studies. Kim et al. (2010) studied the growth and survival of *E. coli* O157: H7, *Salmonella* spp. and *L. monocytogenes* in dairy compost at an initial population of ca. 1 log CFU g^{-1} under greenhouse condition. The pathogen populations increased within first 3 days, followed by rapid decline. After 7 days, the pathogen population stayed stable and showed an obvious tailing effect for all pathogens. This effect may be explained by the fact that more sensitive population of the pathogens was killed by the environmental stresses while a few resistant cells were able to survive and persist under the same stresses. The prolonged survival of pathogens may also result from the stress adaptation phenomenon. Briefly, when the pathogens are exposed to a sublethal stress, they will suffer from a sublethal-cellular injury and temporary cessation of growth (Wesche et al., 2009). However, the pathogens will not be killed under a sublethal stress. The sigma factor, *RpoS,* is believed to be able to control the expression of genes related to stress response in *Salmonella* and *E. coli*. *RpoS* can be induced by environmental stresses and promote the production of stress response proteins (Wesche et al., 2009). Thus, once exposed to sublethal stress, pathogens are able to survive subsequent lethal levels of the same stress better (Rodriguez et al., 2005). In this study, *Salmonella* and *E. coli* O157: H7 were exposed to the stresses of low nutrients/starvation and desiccation once put into greenhouse. After 5 days of storage, it is possible that some of the cells were induced to

have higher resistance due to the stress response mechanisms and were able to survive for extended time.

Moisture content of compost is considered to be an important factor affecting pathogen survival and growth (Sidhu et al., 1999). Rothrock et al. (2012) conducted a greenhouse study to investigate the influence of volumetric water content (VWC) in compost and the absence of clover on survival and growth of *E. coli* O157: H7. They found that *E. coli* O157: H7 survived at significantly lower population in the compost with 45 % VWC treatment as compared to 25 % VWC. In that study, 45% VWC was near the water holding capacity of their samples and the moisture level was maintained throughout the experiment by an automatic system. Thus, the samples were not affected by any dehydration. They suggested that lower moisture content in the compost samples results in an aerobic environment which promotes the survival and persistence of *E. coli* O157: H7, whereas high moisture content in a sample tends to create a fermentative environment which inhibits the bacterial survival. On the other hand, dried cells are expected to have long term survival after adapted to the environment when no further dehydration occurs due to the already low a_w of the sample.

In our study, the initial MC of compost samples were set at 20, 30 and 40% which were the typical moisture levels of finished composts and the compost samples were kept in greenhouse with no additional moisture adjustment. In summer trial, moisture content increased within the first day of storage and decreased considerably slower than samples in late fall trial and winter trial after 5 days. This may result from the high relative humidity (64.4%) maintained in the greenhouse during summer months (Fig 2.1).

Overall, the pathogen in the compost with the same particle size survived better when the initial moisture level in compost was low (Table 2.1), with exceptions of *Salmonella* and *E. coli* O157: H7 in samples with particle size of >1000 μm in summer trial and samples with particle size of <500 μm in winter trial.

For this study, the effect of moisture on pathogen survival can be divided into two stages. In the first 5 days of storage, the rapid water loss in compost samples resulted in a severe dehydration in pathogens. It is well-documented that a fast dehydration process leads to an efficient microbial inactivation (Antheunisse et al., 1979). In our study, the relationship between dehydration rate and pathogen inactivation within 5 days supported that assumption (Table 2.1 and 2.3). After initial rapid water loss in compost samples, pathogens were exposed to constant desiccation stress. Since the pathogen cells have already adapted to the dry condition after 5 days of storage in greenhouse, these cells have developed the ability to survive for extended time in dry compost samples. The mechanisms for pathogen survival in dry environments have been explored (Crowe et al., 1981). When cells are under desiccation stress, they accumulate various solutes such as trehalose, which serve as substitute solutes as well as water replacement agents. The accumulation of trehalose or sucrose occurs in bacterial cells under extreme desiccation situations. Trehalose is a water soluble, non-reducing disaccharide. Studies reported that trehalose is accumulated at a level up to 20% of its dry weight when the cell is under desiccation stress (Crowe et al., 1981), suggesting that trehalose may play a critical role in protecting desiccated pathogenic cells. Trehalose has the ability to interact directly with proteins (Lee et al., 1989) and lipids (Crowe et al., 1981), and stabilizes the cell

structure during desiccation. The function of trehalose can be described by the water replacement hypothesis (Potts, 1994). When cells are stressed by desiccation, trehalose molecules can replace the water position around macromolecules and prevent them from damaging and the water around proteins and between phospholipids in membrane to keep them stable (Potts, 1994; Potts, 2001). By acting as glasses, accumulation of trehalose or other solutes can prevent complete dehydration of cell when temperature is under the melting point of the glass. Besides, in glass state, the metabolism in cell is slowed and water in cell is fixed, making the cell stable (Potts, 1994). The lower overall survival of pathogens in winter trial might be explained by the higher dehydration rate compared to other two trials (Table 2.1). Moisture content of winter trial samples was reduced to less than 10% within 3 days compared to less than 10% within 5 days of late fall trial and summer trial. It also can be noticed that, for the samples with particle sizes of >1000 μm, lower survival of pathogens were observed in higher initial moisture content samples. This may be due to the higher initial moisture content samples caused more rapid dehydration in first 3 or 5 days (Table 2.1).

Temperature is considered as one of the factors influencing pathogen survival and growth in finished compost (Singh et al., 2010; Pietronave et al., 2004). In this study, average temperature in greenhouse was 21.3, 21.7 and 28.4℃ for late fall, winter and summer trials, respectively. Both the highest and lowest temperatures in the greenhouse room were controlled. As a result, the temperature in day time reached the higher limit for all trials as well as the night temperature reached the lower limit for late fall trial and winter trial, which minimized the effect of temperature difference among trials. Based on

SAS analysis of pathogen survival data, season was not considered as a significant factor for *Salmonella* and *E. coli* O157: H7 survival in this study. However, in all trials, the average temperatures were lower than the optimum temperature for *Salmonella* and *E. coli* O157: H7 growth. Pietronave et al. (2004) investigated the survival and growth of seeded *Salmonella* and *E. coli* O157: H7 in finished compost with different indigenous microflora, MC and temperature conditions. Their results demonstrated that in non-sterilized samples with 10% MC, *Salmonella* stored at room temperature had 0.74 log lower survival compared to those stored at 37 ℃. And the difference was smaller when the moisture contents were 40% (0.17 log). The effect can be explained by the fact that when the moisture content was 40% for both samples, the dehydration rate was similar and it was the most effective factor contributing to the inactivation of pathogens. While at low moisture content, the water activity became considerably stable and temperature became the predominant factor in this situation. In this study, there was a higher *Salmonella* reduction difference in the samples with 20% MC among three seasons than in the samples with 30% MC. For example, the *Salmonella* log reductions within 5 days were 3.19, 2.93 and 2.14 for late fall, winter and summer trials with initial MC of 40% and particle size of >1000 μm, respectively. However, when the initial MC was 20%, the log reductions within 5 days were 1.39, 2.88 and 1.95 for late fall, winter and summer trials with particle size of >1000 μm, respectively. Our results suggest that in the samples with lower initial MC, the temperature effect is more significant ($P<0.05$) than in the samples with higher initial MC. Himathongkham et al. (1999) investigated the survival of *Salmonella* and *E. coli* in cow manure and found that they have a greater survival at 20 ℃

compared to 4 or 37 ℃. This may have resulted from the high moisture content from their experiment, which was considerably stable (80-90%) throughout the experiment; thus the effect of moisture stress is minimized. Whereas, the constant low temperature which below the optimum growth temperature of *Salmonella* and *E. coli* O157: H7 decreases the enzyme activity and retards the metabolism of pathogens. And this can results in less inactivation from environmental stresses.

The influence of particle sizes of compost samples on the survival of pathogens was examined in this study. For all samples, compost with particle size of >1000 μm had better survival of both *Salmonella* and *E. coli* O157: H7 than those in 500-1000 μm and <500 μm samples with the same moisture content (Table 2.1 and 2.2). Pathogens died off the quickest in the compost samples with particle size of < 500 μm although the statistic analysis showed some of the log reduction differences for *Salmonella* and *E. coli* O157: H7 were not significant for the same treatment (Table 2.1 and 2.2).

There are several possible explanations for the differences in pathogen survival rates between large and small particles. As compared to samples with the smaller size, the samples with larger size have more variations in terms of material type, nutrients, texture and shape. For example, wood shavings and plant materials are mainly in the large particle portion and they have better water holding capacity inside the material. This may lead to nutrient and pathogens shielded between or inside particles. Also, the aggregation of samples with smaller particle size may create a fermentative environment which lead to inhibition of pathogen due to the space between each particle is minimized and the particles are more compacted. The smaller the particle is, the more surface/volume ratio it

has. Also, the smaller the particle is, the simpler the surface it has as compared to larger particles. Consequently, more rapid water loss occurred in smaller compost particles as observed in this study (Table 2.1). In addition, as the particle size decreases, the air holding capacity reduces, leading to more temperature fluctuation during day and night.

Although no studies have examined the pathogen survival in compost as affected by particle size, several studies have determined the relationship between the matrix size and the pathogen survival in soil sediments. Burton et al. (1987) tested the survival of 4 pathogens in fresh water sediments. The samples were monitored in a flow chamber for a period of 14 days. They found that the only sediment property associated with survival of pathogens was particle size. *E. coli* and *S.* Newport survived longer in samples with high concentration of clay (smaller particle) than in samples with high concentration of sand (large particles). However, they suggested that the influence of clay may result from the higher organic matter and nutrient supported the survival of pathogen. Similarly, Grimes (1980) reported that pathogens survive longer in high clay sediment compared to high sand sediment in Mississippi river. However, the result failed to prove the particle size was a factor to the survival. Howell et al. (1996) conducted a laboratory study to determine the survival of fecal bacteria in sediment of agriculture water. They reported that the mortality rate of fecal bacteria decrease as the particle size decrease. All above samples were in aqueous environment, whereas our samples were exposed to air. The drastic difference in the environment the pathogens were subjected to may explain the different results between ours and above studies.

Particle size is a key factor to the pathogen carriage and transmission. Aerosolized pathogen transmission can be classified into 2 categories: droplet or airborne (Gralton et al., 2011). Droplet is mainly defined as particles that are able to settle down quickly from the contamination source. Airborne are defined as particles that can stay suspended in the air and transmit to longer distances. On compost sites, aerosols can be generated from the compost surfaces and dispensed as small airborne aerosols. Also, the compost particles with pathogens can travel via 'droplet transmission'. The droplet transmission is mainly decided by the location of contamination source, and as particle size increases, the gravitational settling increases, resulting in decrease of the distance the particle can travel. To measure the airborne pathogen on compost site, Fischer et al. (2008) conducted a 3 year study of composting facilities about emission and dispersal of microorganisms. They revealed that airborne microorganisms reached highest levels during compost turning, e.g. 2.4×10^6 CFU /m^3 for thermophilic actinomycetes and about 10^5 CFU /m^3 for other microorganisms. Lighthart et al. (1987) used virus as a sample to set up a model of airborne microbial survival with certain environmental variables. The multiple regression illustrates the potential importance of wind as a dilution and survival factor, which means the microbes are diluted by a factor of 10,000 in the first 30 m downwind from the source. To evaluate the risk of pathogen contamination of fresh produce due to aerosol from composting site, further study is needed to determine how far these particles investigated in this research can travel in the air.

Conclusion

This study reported that pathogen survival in finished compost samples was affected by initial moisture content, temperature and particle size under greenhouse conditions. Overall, compost samples with larger particle sizes support pathogen survival for extended time as compared with compost samples with small particle sizes. For the same size particle, the pathogen survived better in compost with low moisture content than higher moisture content. Under greenhouse conditions, higher dehydration rate was a critical factor which contributes to the initial rapid inactivation of pathogens esp. in late fall and summer trials. Due to the temperature limits of greenhouse facility, the seasonal effects on pathogen survival were mainly decided by the relative humidity which influences the dehydration rate of the compost samples or UV exposure.

Since this study was conducted under greenhouse conditions, the results may not reflect the actual situation of outdoor compost storage in composting facilities. But it gave some valuable information about the possibilities of pathogen survival in finished compost samples as affected by particle size, moisture content and seasonality. Since pathogens survived on compost surfaces during storage may disseminate into nearby produce fields via aerosols. The surface of finished compost should be treated with physical covering or prevented from being blown away from wind. Also, the composting facilities should be located away from fresh produce farms, as the transmission of compost particles and generated bioaerosols via air happens. However, to reflect the real situation of composting facilities, further studies are needed to investigate the ability and risk of the transmission of compost with different particle sizes in open environments.

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Figure Legends

Fig 2.1 Greenhouse temperature, relative humidity and light intensity in a typical day. The data were recorded hourly by the remote monitoring system. For each trial, data of every experimental day were averaged.

Fig 2.2 Changes in moisture content (A) and populations of *Salmonella* (B) in compost with 40% initial MC as affected by particle sizes (late fall trial). Data were expressed as mean ±SD of two trials and SD were represented by error bars.

Fig 2.3 Changes in moisture content (A) and populations of *Salmonella* (B) in compost with 30% initial MC as affected by particle sizes (late fall trial). Data were expressed as mean ±SD of two trials and SD were represented by error bars.

Fig 2.4 Changes in moisture content (A) and populations of *Salmonella* (B) in compost with 20% initial MC as affected by particle sizes (late fall trial).

Fig 2.5 Changes in moisture content (A) and populations of *Salmonella* (B) and *E. coli* O157: H7 (C) in compost with 40% initial MC as affected by particle sizes (winter trial).

Fig 2.6 Changes in moisture content (A) and populations of *Salmonella* (B) and *E. coli* O157: H7 (C) in compost with 30% initial MC as affected by particle sizes (winter trial).

Fig 2.7 Changes in moisture content (A) and populations of *Salmonella* (B) and *E. coli* O157: H7 (C) in compost with 20% initial MC as affected by particle sizes (winter trial).

Fig 2.8 Changes in moisture content (A) and populations of *Salmonella* (B) and *E. coli* O157: H7 (C) in compost with 40% initial MC as affected by particle sizes (summer trial).

Fig 2.9 Changes in moisture content (A) and populations of *Salmonella* (B) and *E. coli* O157: H7 (C) in compost with 30% initial MC as affected by particle sizes (summer trial).

Fig 2.10 Changes in moisture content (A) and populations of *Salmonella* (B) and *E. coli* O157: H7 (C) in compost with 20% initial MC as affected by particle sizes (summer trial).

Table 2.1 Pathogen reduction and dehydration rate of compost samples as affected by particle sizes, initial moisture and seasonality.

Season	Initial MC	Particle size (μm)	log reduction in first 5 days		Dehydration rate in 5 (3) days (%/d) ***
			Salmonella	*E. coli* O157: H7	
Late fall	40%	>1000	A3.19±0.44a*	NT**	A6.68±0.40****
		<1000	A3.16±0.39	NT	A7.39±0.01
	30%	>1000	A1.80±0.18b	NT	B4.02±0.25
		500-1000	A2.35±0.69a	NT	A5.26±0.40
		<500	A2.74±0.46a	NT	A5.50±0.45
	20%	>1000	A1.39±0.25b	NT	A2.43±0.35
		500-1000	A1.70±0.04a	NT	A3.14±0.51
		<500	A1.78±0.35a	NT	A3.45±0.41
Winter	40%	>1000	A2.93±0.37a	A2.20±0.30a	A6.44(10.25)±0.27
		<1000	A3.36±0.06	A2.84±0.42	A6.81(10.78)±0.31
	30%	>1000	B2.33±0.22a	C1.28±0.10b	A4.48(7.60)±0.57
		500-1000	A3.23±0.33a	B2.05±0.17a	A4.99(8.04)±0.14
		<500	A3.68±0.09a	A2.68±0.17a	A4.90(7.93)±0.12
	20%	>1000	A2.88±0.21a	A2.11±0.27a	A2.49(3.64)±0.32
		500-1000	A3.42±0.85a	A3.07±0.48a	A3.03(4.75)±0.23
		<500	A3.66±1.19a	A3.11±0.57a	A3.07(5.12)±0.13
Summer	40%	>1000	B2.14±0.07a	A2.08±0.16a	B3.10±0.68
		<1000	A3.17±0.02	A2.48±0.06	A6.16±0.18
	30%	>1000	A2.35±1.20a	A2.14±0.89a	B1.69±0.71
		500-1000	A2.61±1.13a	A2.40±1.02a	A3.82±0.15
		<500	A2.84±0.62a	A2.54±0.76a	A4.70±0.14
	20%	>1000	A1.95±1.18a	A1.82±1.51a	C-0.23±0.03
		500-1000	A1.93±1.12a	A2.19±1.01a	B1.51±0.19
		<500	A2.10±1.08a	A2.53±0.91a	A2.62±0.24

*Data are expressed as means±SD of two trials. Means with different upper case letters in the same column within each season and MC are significantly different ($P < 0.05$) according to the LSD test. Means with different lower case letters in the same column with the same particle size in each season are significantly different ($P < 0.05$) according to the LSD test.
**NT, not tested.
***In winter trial only, the dehydration rate is significantly different between day 3 and 5.
****Dehydration rate is expressed as moisture content reduction/days of storage.

Table 2.2 Pathogen reduction in 3 trials as affected by MC and particle size of compost

Trial	Initial MC**	Particle size (μm)	log reduction in 30 days	
			S. Typhimurium	*E. coli* O157: H7
Late fall	40%	>1000	A3.92±0.70a*	NT***
		<1000	A4.57±0.98	NT
	30%	>1000	A2.66±0.00ab	NT
		500-1000	AB3.17±0.29a	NT
		<500	B3.47±0.06a	NT
	20%	>1000	A1.80±0.32b	NT
		500-1000	A2.18±0.51a	NT
		<500	A2.34±0.10b	NT
Winter	40%	>1000	A4.70±0.98a	A2.95±1.02a
		<1000	A4.88±0.57	A3.55±0.93
	30%	>1000	A2.98±0.32a	B1.95±0.03a
		500-1000	A4.50±1.03a	AB2.68±0.49a
		<500	A5.04±0.92a	A3.69±0.47a
	20%	>1000	B3.90±0.16a	B2.88±0.24a
		500-1000	A5.23±0.49a	A4.30±0.29a
		<500	A6.10±0.10a	A4.57±0.12a
Summer	40%	>1000	A3.55±0.57a	A3.35±0.28a
		<1000	A4.07±0.42	A3.72±0.33
	30%	>1000	A3.08±1.29a	A3.04±0.61a
		500-1000	A3.27±1.42a	A3.00±0.68a
		<500	A3.55±0.99a	A3.27±0.58a
	20%	>1000	A2.69±1.10a	A2.59±1.20a
		500-1000	A2.65±0.88a	A3.11±1.11a
		<500	A2.76±0.78a	A3.52±0.99a

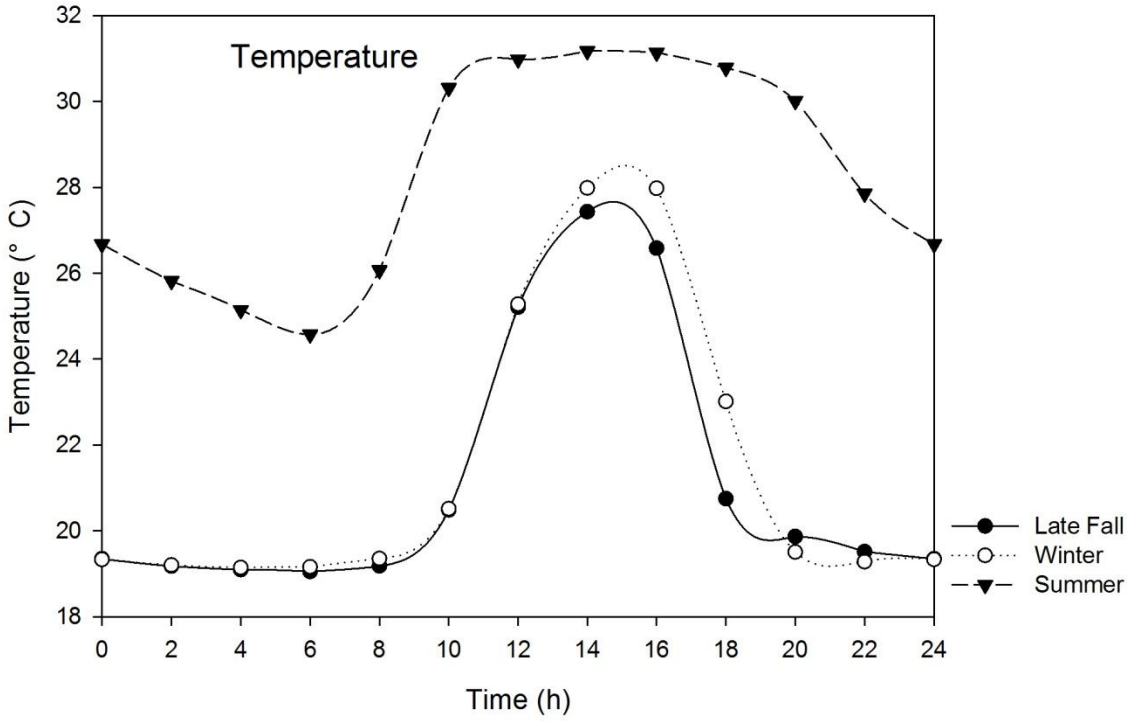
*Data are expressed as means±SD of two trials. Means with different upper case letters in the same column within each season and MC are significantly different ($P < 0.05$) according to the LSD test. Means with different lower case letters in the same column with the same particle size in each season are significantly different ($P < 0.05$) according to the LSD test.
**Initial moisture content, which is not adjusted during the storage.
***NT, not tested.

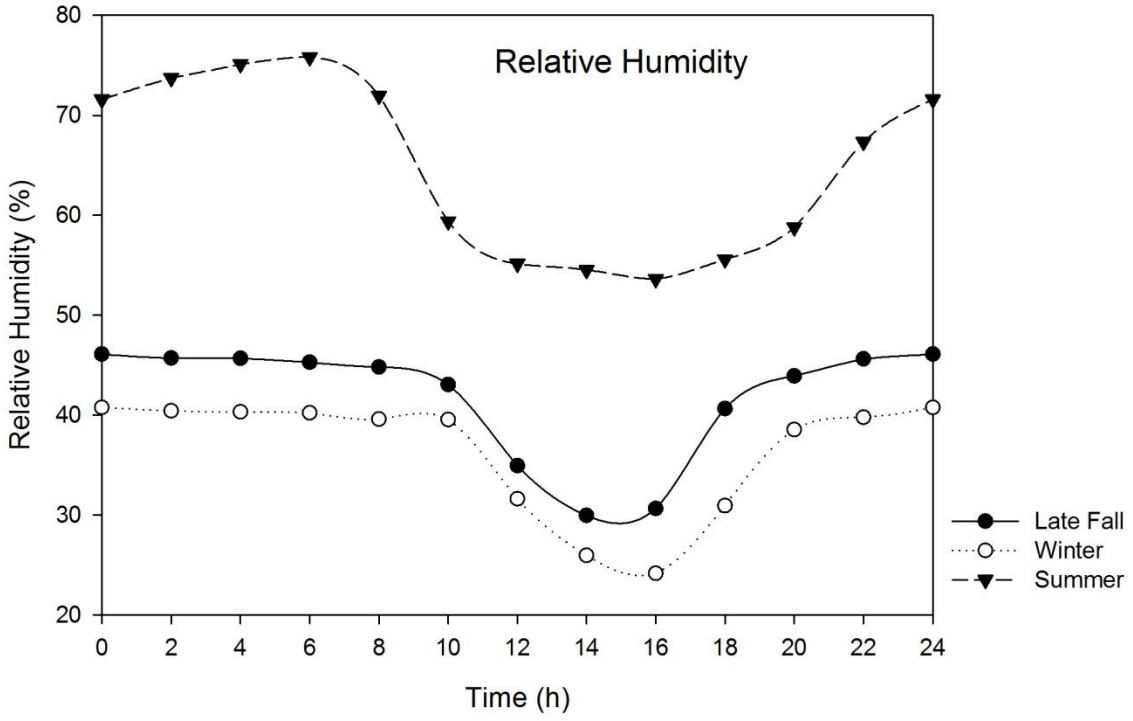
Table 2.3 The correlation of pathogen reduction and dehydration rates of compost samples in different seasons

Trial	Pathogens	Correlation	Regression		
			R^2*	X variable**	P-value***
Late fall	*Salmonella*	0.98	0.96	0.39	0.00
Winter	*E. coli* O157: H7	-0.12	0.01	-0.04	0.77
	Salmonella	-0.05	0.00	-0.01	0.90
Summer	*E. coli* O157: H7	0.79	0.63	0.10	0.02
	Salmonella	0.90	0.80	0.20	0.00

*R^2, represents the ability of dehydration rate to predict the log reductions of pathogens.
**X variable, represents the value log reduction of pathogen change when the dehydration rate rise by 1%/d.
***P-value represents the significance of X variable.

Fig 2.1 Greenhouse temperature, relative humidity and light intensity in a typical day





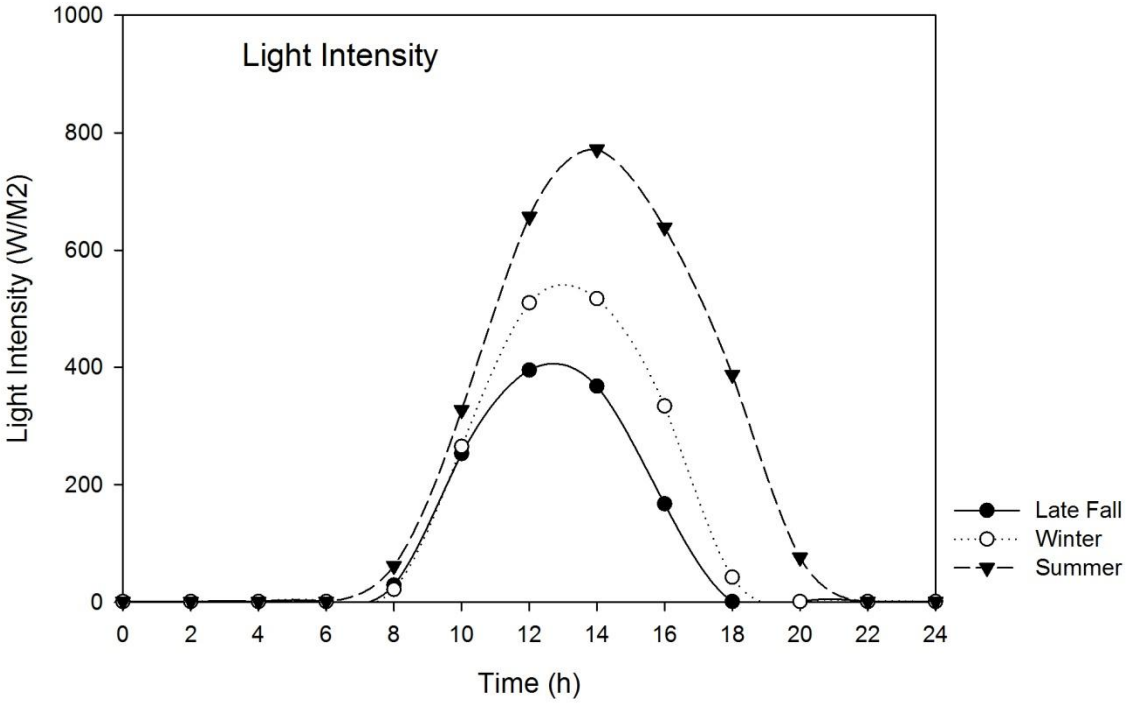

Light Intensity
Light Intensity (W/M2)
1000
800
600
400
200
0
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (h)
Late Fall
Winter
Summer

Fig 2.2 Changes in moisture content (A) and populations of *Salmonella* (B) in compost with 40% initial MC as affected by particle sizes (late fall trial).

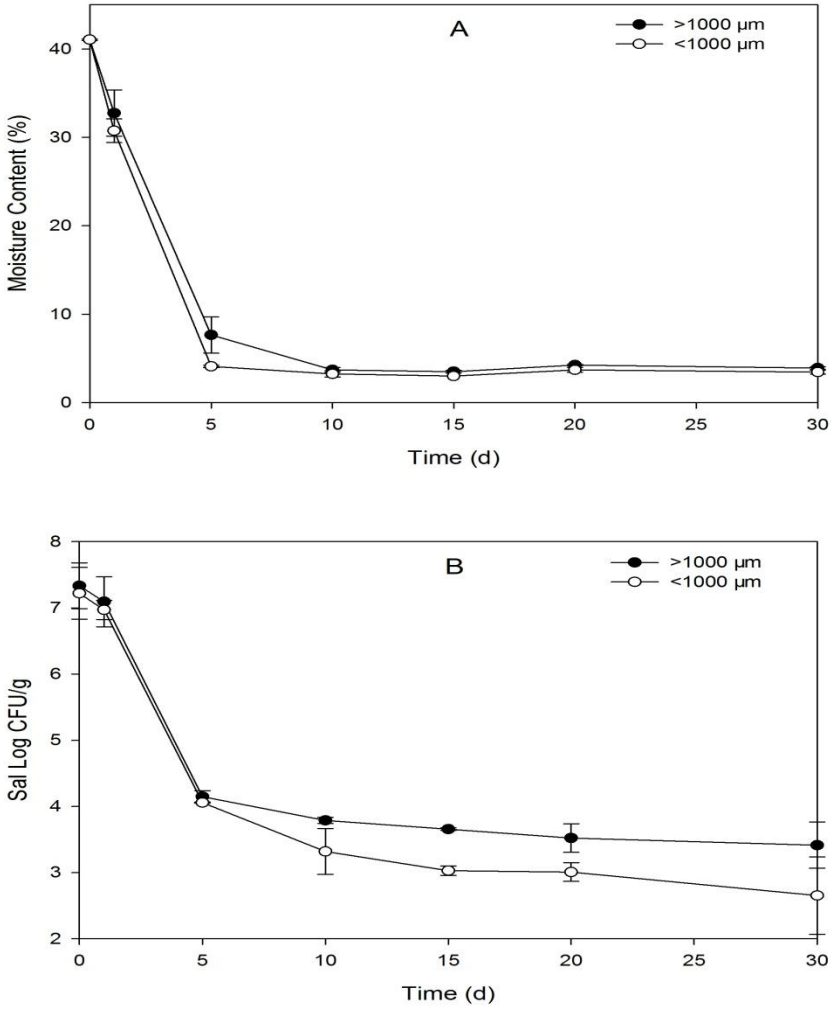


Fig 2.3 Changes in moisture content (A) and populations of *Salmonella* (B) in compost with 30% initial MC as affected by particle sizes (late fall trial)

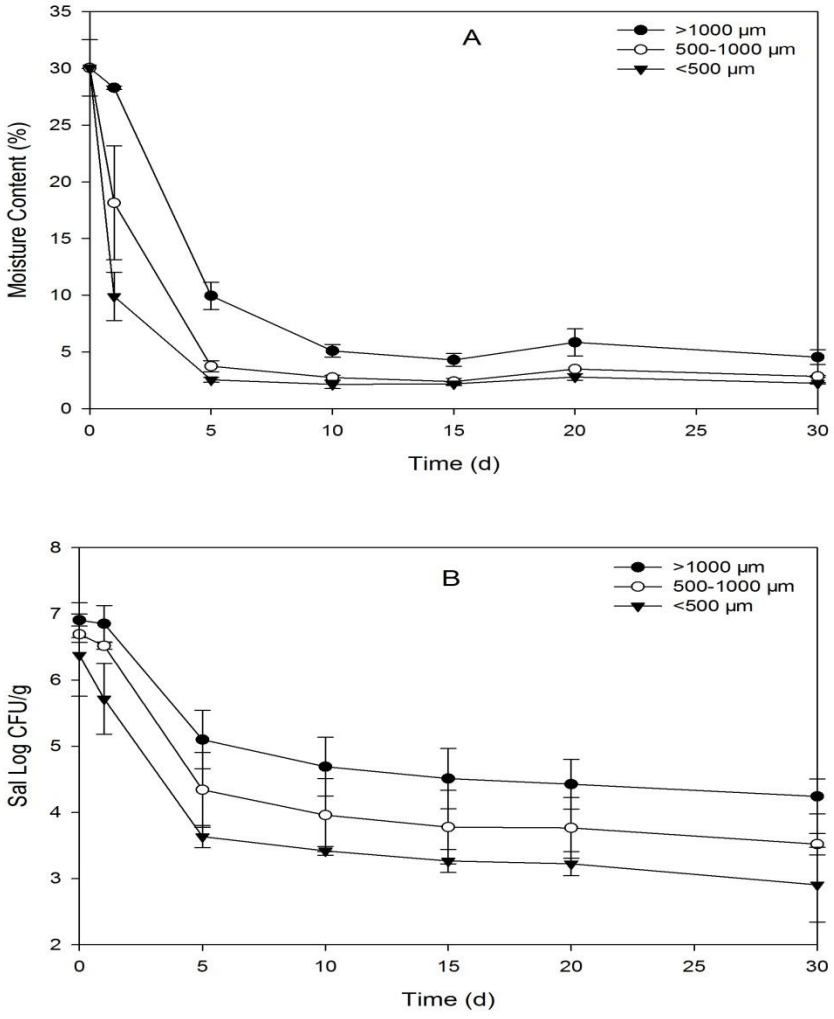


Fig 2.4 Changes in moisture content (A) and populations of *Salmonella* (B) in compost with 20% initial MC as affected by particle sizes (late fall trial)

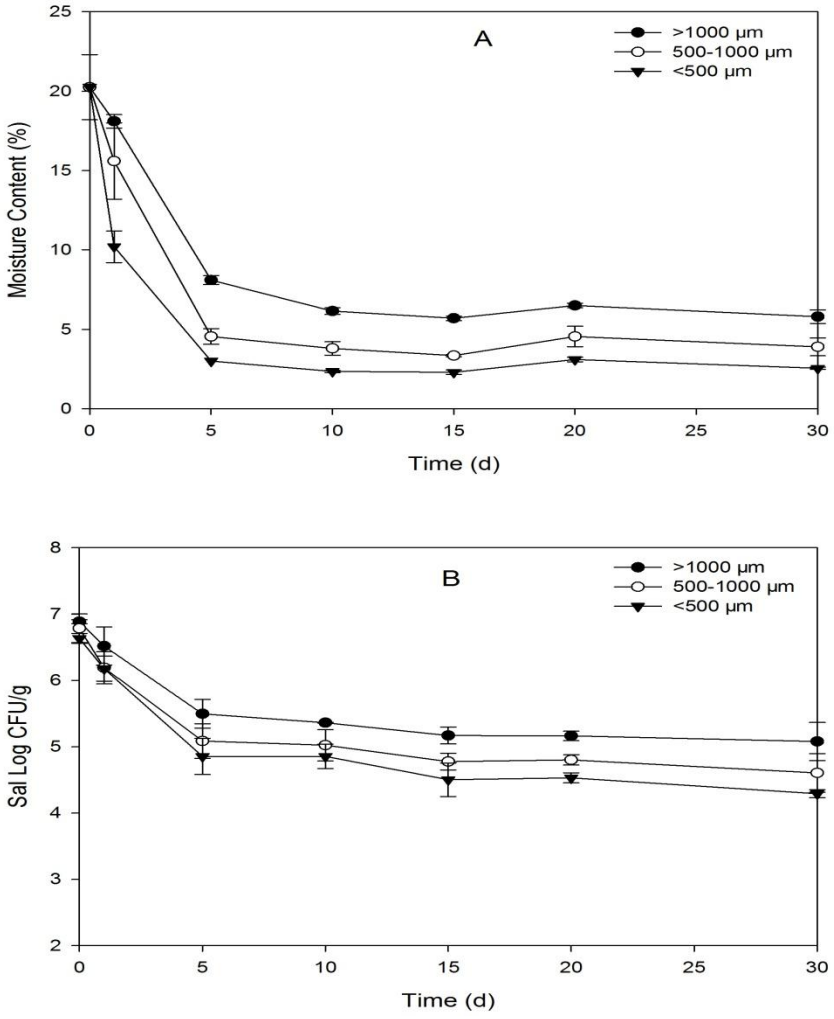


Fig 2.5 Changes in moisture content (A) and populations of *Salmonella* (B) and *E. coli* O157: H7 (C) in compost with 40% initial MC as affected by particle size (winter trial)

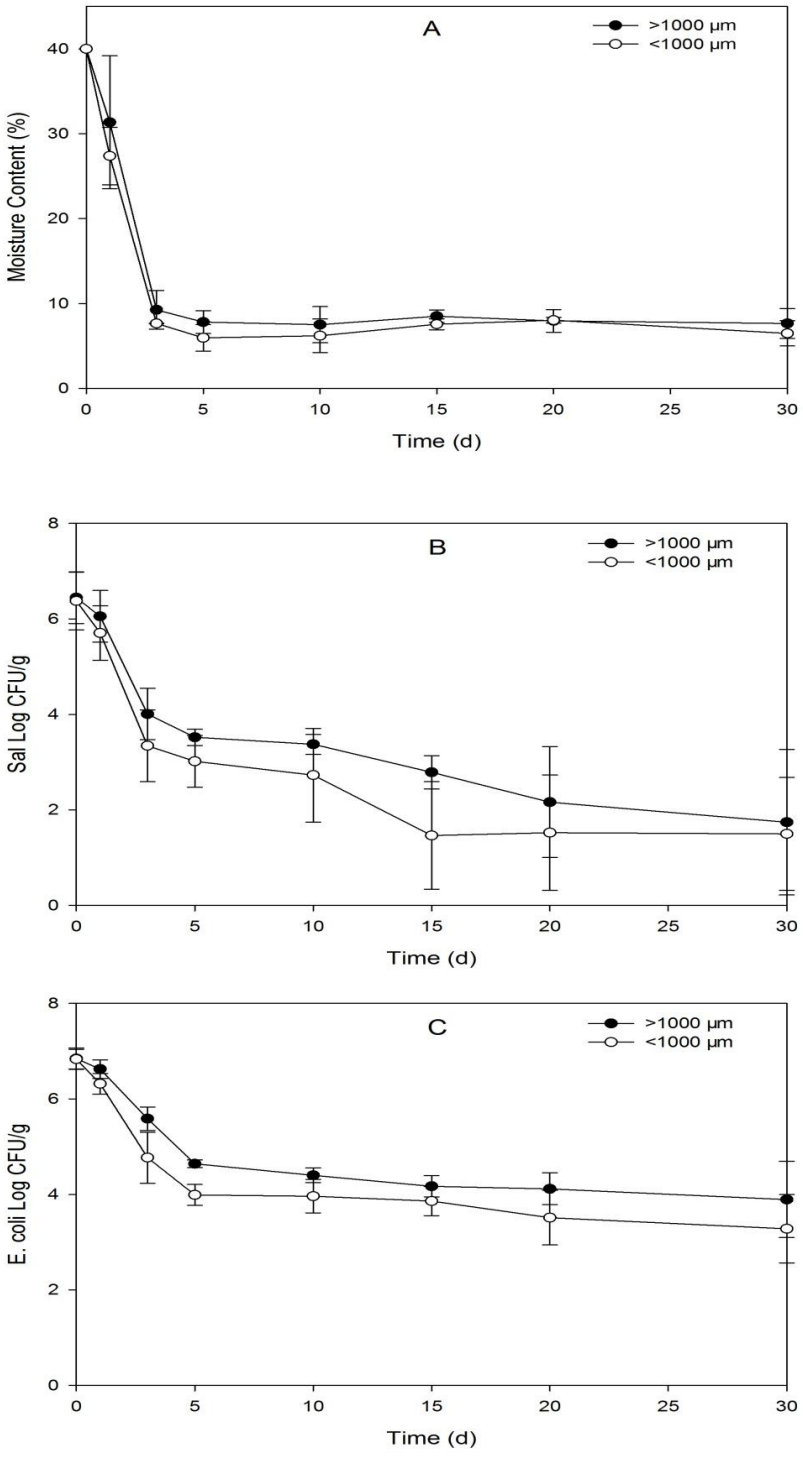


Fig 2.6 Changes in moisture content (A) and populations of *Salmonella* (B) and *E. coli* O157: H7 (C) in compost with 30% initial MC as affected by particle sizes (winter)

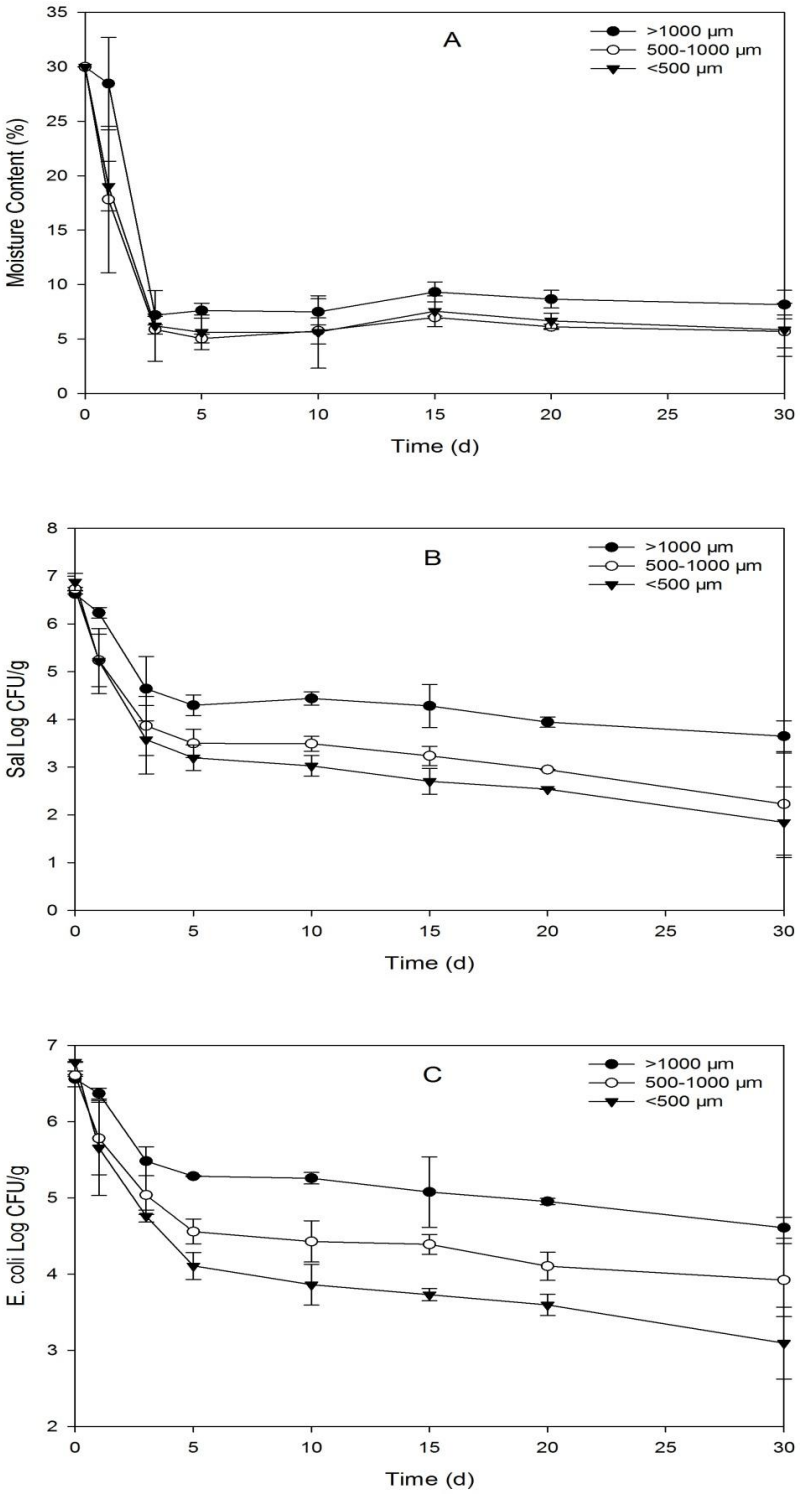


Fig 2.7 Changes in moisture content (A) and populations of *Salmonella* (B) and *E. coli* O157: H7 (C) in compost with 20% initial MC as affected by particle sizes (winter)

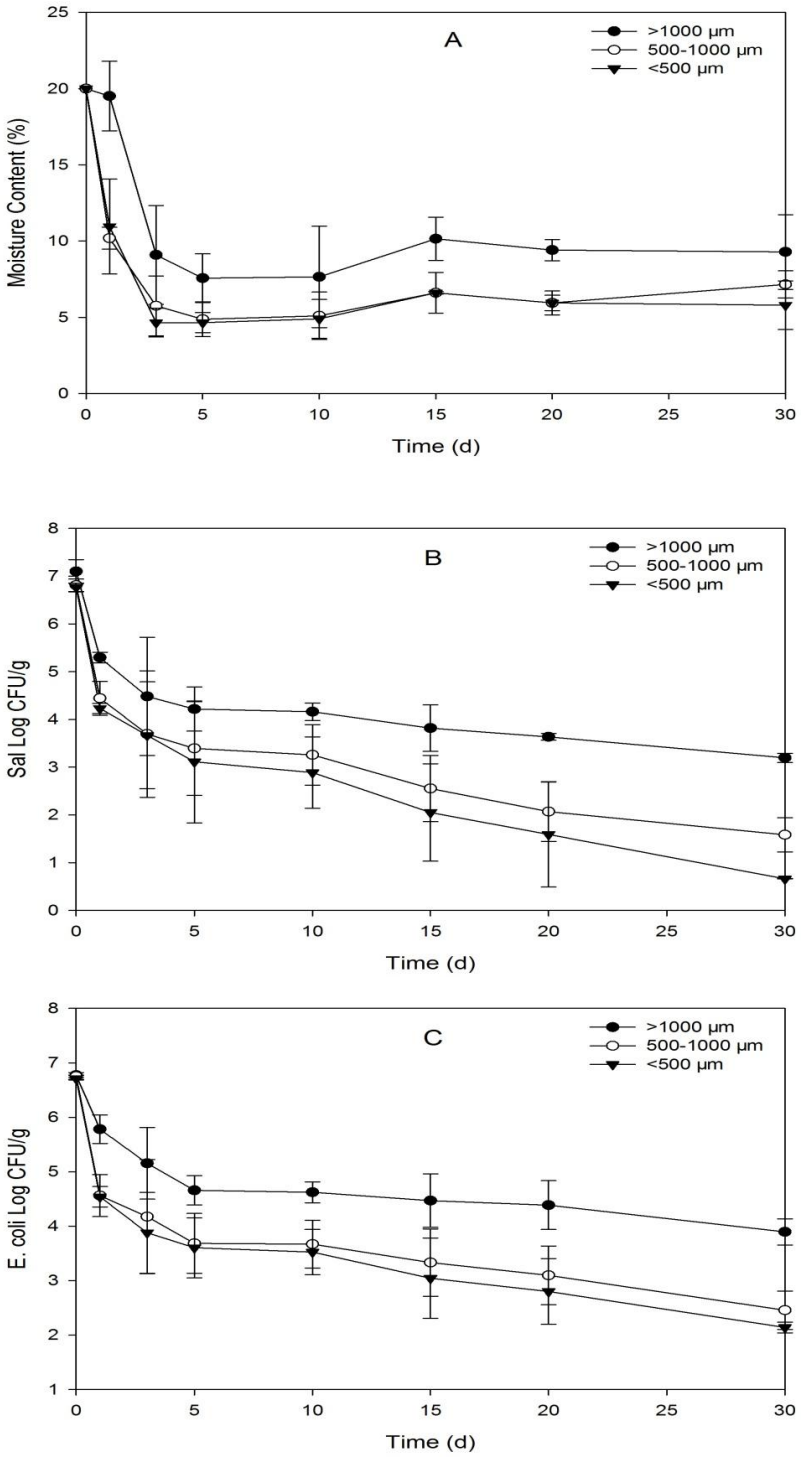


Fig 2.8 Changes in moisture content (A) and populations of *Salmonella* (B) and *E. coli* O157: H7 (C) in compost with 40% initial MC as affected by particle sizes (summer)

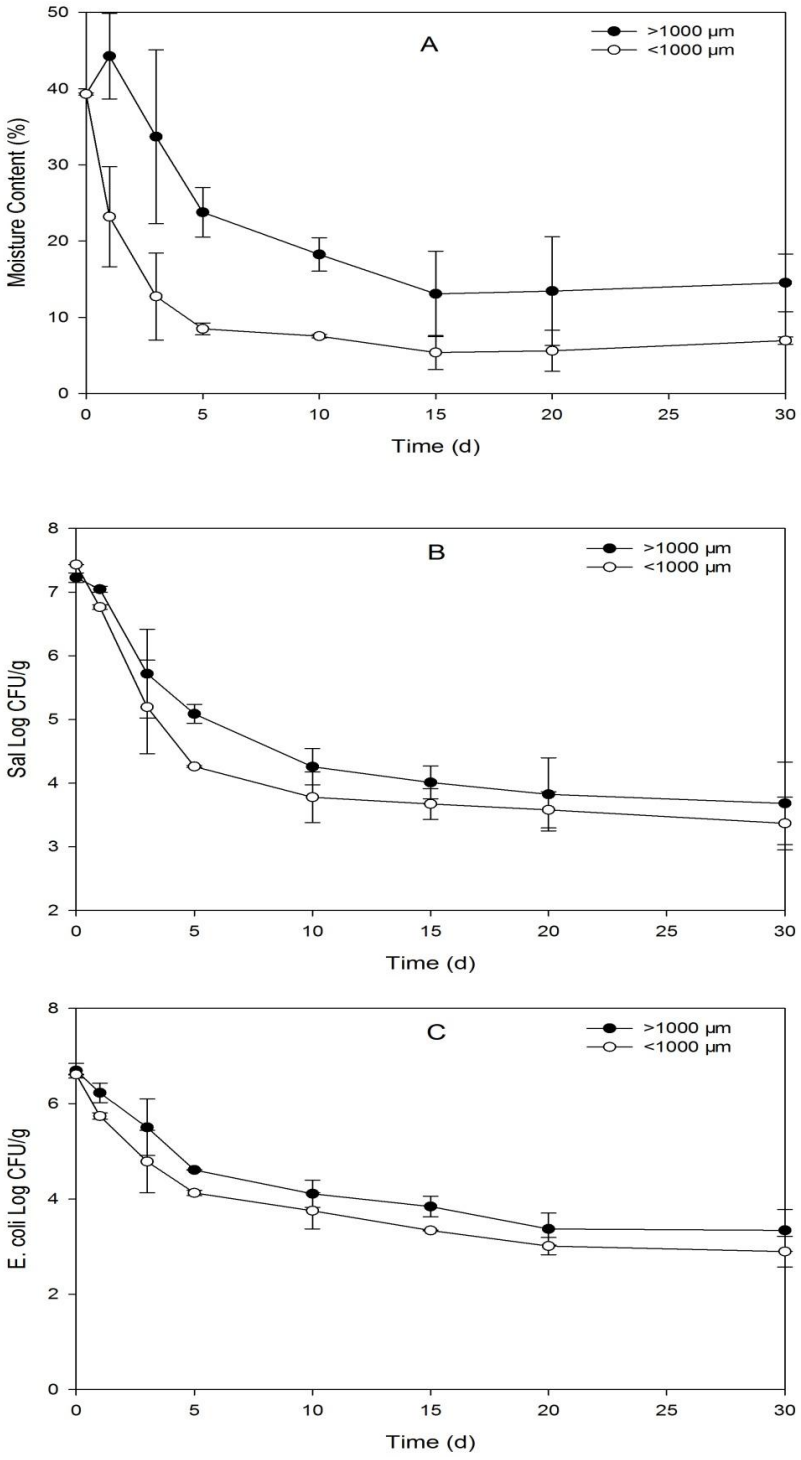


Fig 2.9 Changes in moisture content (A) and populations of *Salmonella* (B) and *E. coli* O157: H7 (C) in compost with 30% initial MC as affected by particle sizes (summer)

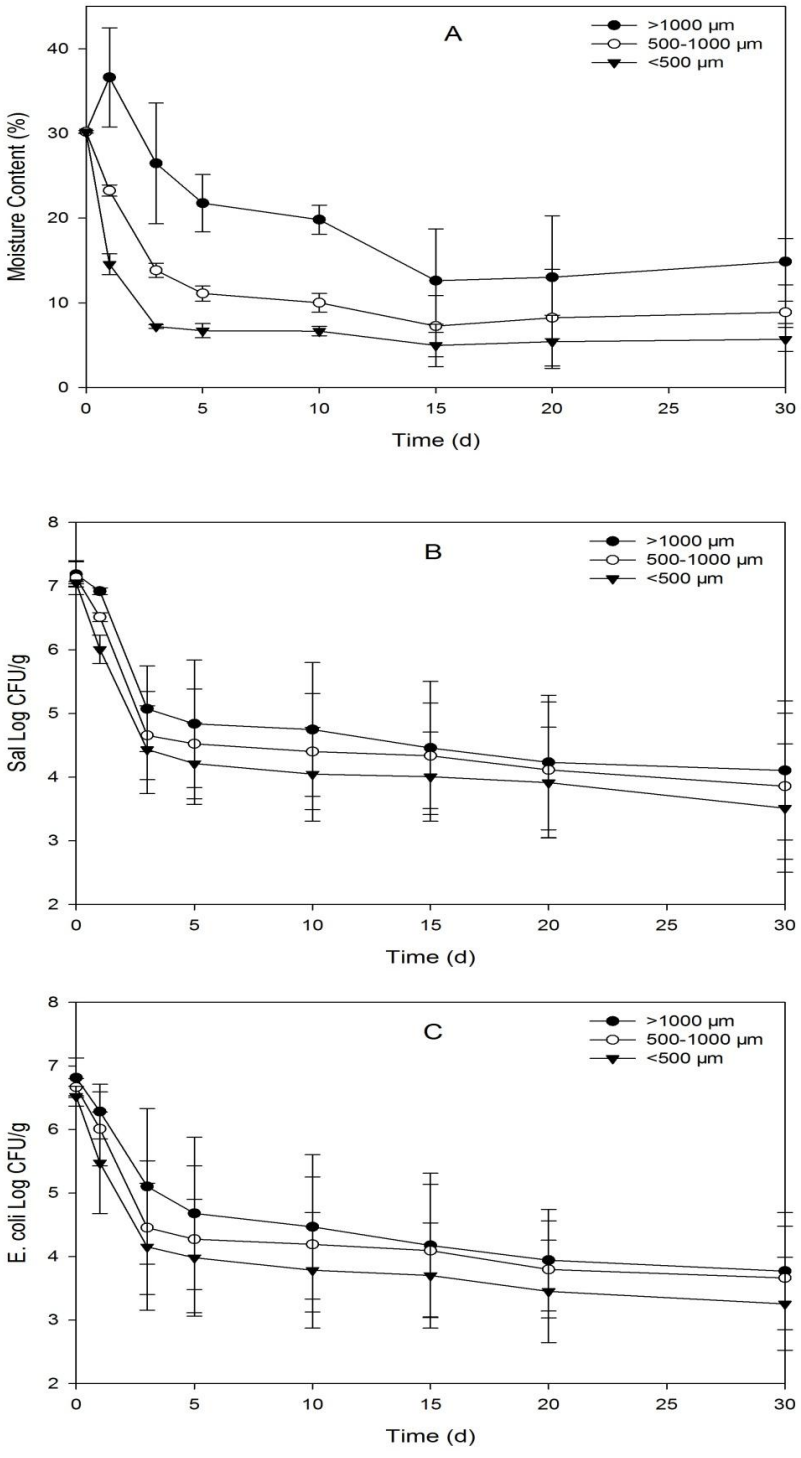
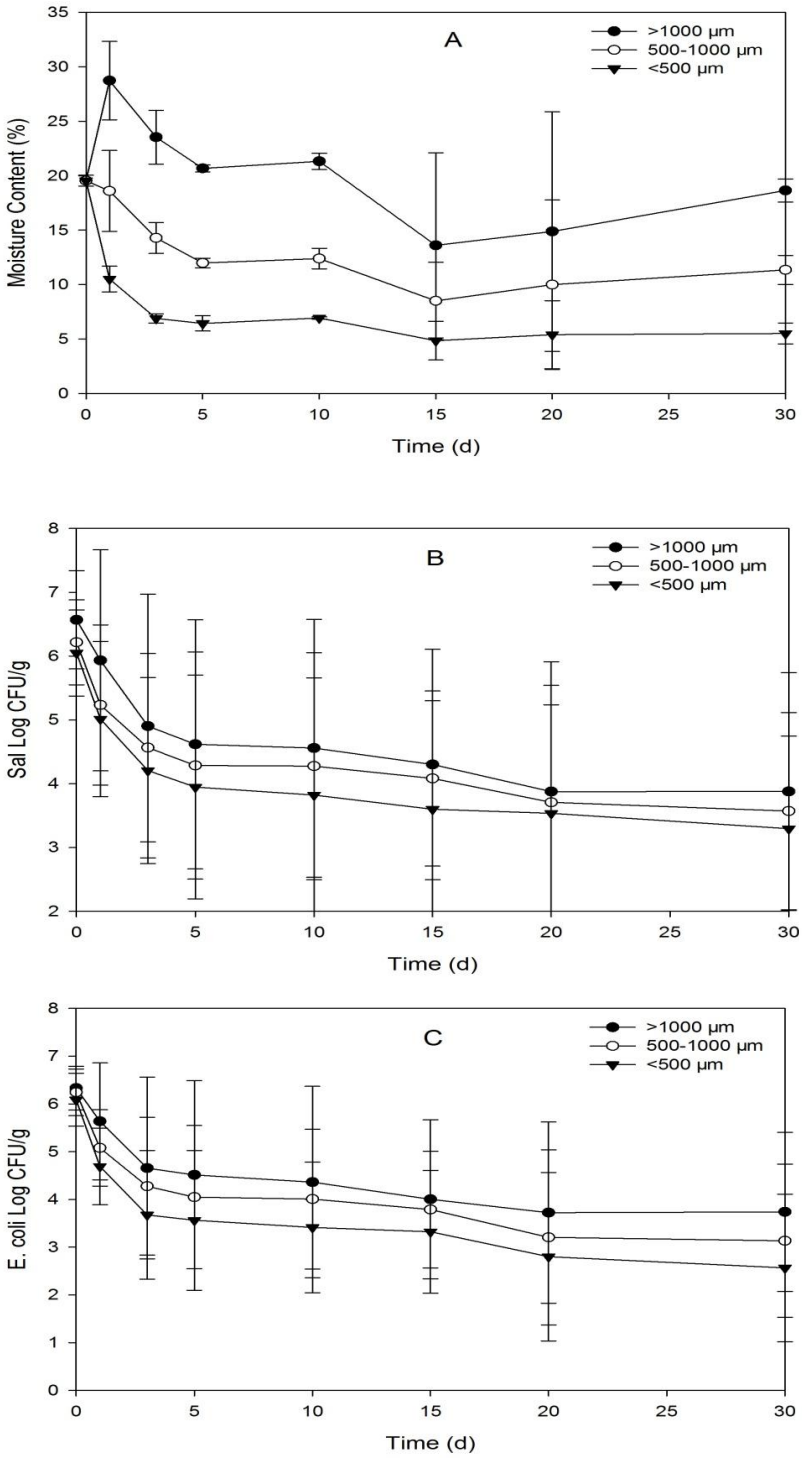


Fig 2.10 Changes in moisture content (A) and populations of *Salmonella* (B) and *E. coli* O157: H7 (C) in compost with 20% initial MC as affected by particle sizes (summer)



CHAPTER THREE

DEVELOPING A MICROSCOPIC METHOD TO VISUALIZE INTERACTION OF *ESCHERICHIA COLI* O157: H7 CELLS WITH COMPOST PARTICLES

Abstract

Our previous study has revealed the varied survival of *Escherichia coli* O157: H7 and *Salmonella* spp. in compost with different particle sizes. In order to understand the pathogen survival strategies, microscopic observations of the interactions between bacterial cells and compost particles were explored using several approaches in this study. First, the morphological and surface characteristics of compost particles with different particle sizes were analyzed by a surface profiler. Then finished compost samples were inoculated with green fluorescence proteins (GFP)-labeled *E. coli* O157 H7 and observed under a fluorescence microscope. Since the sensitivity of fluorescence microscope is negatively affected by broad spectra and high intensity from autofluorescence of compost matrix, spectral analysis and unmixing approach were applied to separate overlapped spectra of target signal and the background autofluorescence. The emission wavelength and intensity of both GFP and autofluorescence from compost were measured and compared statistically. However, spectra analysis could not provide information about clear location of individual cells in compost matrix visually. Our next approach was to use immunofluorescence (IF) protocol for enhancing the GFP signal. IF results showed that GFP in *E. coli* O157: H7 and autofluorescence of compost can be differentiated both visually and quantitatively according to the signal intensity. Different blocking agents and concentrations of

antibodies were tested but no significant improvements on enhancing target signal or reducing background fluorescence were observed from these modifications. Next, quantum dot conjugates (Qdots) were used and the results showed that Qdots are effective treatment for enhancing signals of GFP-labeled *E. coli* O157 H7 in compost matrix.

Our results suggested that GFP expressed by bacterial cells is not sufficient to differentiate target cells from background autofluorescence in compost. Spectra analysis can separate overlap emissions from GFP-labeled pathogen and compost statistically, however, amplification of GFP signal by immunofluorescence methods, esp. Qdots, can improve the detection of target cells in compost matrix.

Introduction

Composting process has been widely used for animal waste treatment. The final product of composting process is a nourishing, stable and humus-like material (Dickson et al., 1991). Studies have reported that pathogens are usually eliminated if composting is handled properly by following the EPA or USDA guidelines (Ceustermans et al., 2007; Shepherd et al., 2007; Lung et al., 2001; Hess et al., 2004). However, it is possible that pathogens can survive, recolonize and regrow on compost heap surfaces to a hazardous level (Kim et al., 2009, 2010; Zaleski et al. 2005; Pietronave et al., 2004). Our previous study has reported different survival of *E. coli* O157: H7 and *Salmonella* in compost as affected by different particle sizes (Chapter 2). Therefore, it's interesting to develop a

visual method to determine how these pathogens interact with compost matrix in order to fully understand the survival strategies of human pathogens in compost.

Compost is a heterogenic and complex sample matrix for microscopic observation. Different components such as sand, soil or wood chips may exist in the finished compost, which results in the heterogenic sample properties in terms of water holding capacity, stain ability and surface area. Furthermore, the autofluorescence from compost material seriously affect the microscopic observation of fluorescence emission from fluorescence labeled bacteria in compost matrix (Carlsson et al., 1997). Thus, observation of living microorganisms in compost samples under microscopes is very challenging.

In order to view the interactions between bacterial cells and compost particles, the cells have to be labeled with a marker or stained. Green fluorescent protein (GFP) is an excellent marker for live cell imaging. The GFP was cloned from a jellyfish and used for labeling structures within cells and tissues (Charlfie et al., 1994). Studies have shown that GFP is a valuable tool to study microbial activities and communities in the environmental samples (Lagendijk et al., 2010; Edwards, 2009). Live cell imaging is benefited from multiple labeling fluorescence techniques for its ability to achieve identification of multiple molecular or structural components simultaneously (Kho et al., 2008; Medina et al., 2002).

The major disadvantage of multichannel detection method in live cell imaging is that the broad (ca. 80 nm) and overlapping emission spectra can affect the signal sensitivity of the target (Carlsson et al., 1997). The emission fluorescence signals need to be discarded in a great amount to achieve a reliable spectral separation. Thus,

conventional filter-based multi-labeling techniques have a limitation of simultaneous detection of 4 fluorophores (Waggoner et al., 1989). On the other hand, inorganic fluorescent probes, such as quantum dots, have the advantage of significantly narrow and symmetric emission spectra (Michalet et al., 2005) and can overcome above limitations.

Spectral imaging followed by linear unmixing is a technique that has been designed for separating overlapping fluorescence signals from fluorescence proteins (Zimmermann et al., 2003; Kho et al., 2008). This technique can be used to investigate images captured by epi-fluorescence microscope as well as confocal microscope. It is used when careful selection of excitation sources and emission filters fail to process the sample signals (Anderson et al., 2006). This technique utilizes the emission spectral curve data from individual signals to mathematically decompose the mixed sample emission spectra (Zimmermann et al., 2005; Berg et al., 2004). Thus, the relative contribution of each signal needs to be available as a spectral profile. The major advantage of this application is that the increased signals can be detected in one time and the data can be analyzed later in a short time without any additional sample treatment. Wolf et al. (2005) have applied this technique to image the phycobiliprotein within cyanobacteria.

Immunofluorescence (IF) microscopy is a widely used technique primarily for the fixed samples. This technique utilizes the specific affinity of the antibody coupled with fluorescence dyes to the target antigens. It is a very sensitive method that uses a fluorescence microscope to visualize the location of a specific protein or cell (Janse et al., 2009). Generally, IF microscopy uses a primary antibody to recognize and bind to the

target protein. Then, a fluorescently-labeled secondary antibody is used to bind to the primary antibody (Welter et al., 2002). Moreover, other applications are used to overcome background fluorescence. For example, blocking agents and the application of 'nonsense' probes are used for eliminating non-specific binding (Palasubramaniam et al., 2008), whereas image analysis models are able to subtract broad emission spectrum of background autofluorescence from probe fluorescence (Whiley et al., 2011).

In this study, several microscopic methods were explored to visualize GFP-labeled *E. coli* O157: H7 in the finished compost, and methods to minimize the autofluorescence in compost and enhance GFP signal intensity were applied and compared.

Materials and Methods

Preparation of the finished compost. The commercial Black Kow® dairy compost (Black Gold Compost Company, Oxford, FL, USA) was used in this study. It was purchased from a local Lowe's store and kept at room temperature. As stated on the label, this product has 0.5% total nitrogen, 0.5% available phosphate (P_2O_2), 0.5% soluble potash (K_2O), and no more than 1% chlorine. The finished compost sample was dried under a hood until the moisture content was reduced to less than 20%. This dried sample was then screened by a sieve (sieve pore size, 3 × 3 mm) to a size of less than 3 mm in diameter. Initial moisture content of the finished compost sample was measured with a moisture analyzer (Model IR-35 Infrared analyzer; Denver Instrument, Denver, CO).

Morphological and surface analysis of compost particles by surface profiler. The moisture content of the commercial finished compost was adjusted to 20% with sterile tap water. Without inoculation, the above compost samples were sieved into 3 portions with particle sizes of >1000, 500 -1000 and <500 μm using standard sieves (#18 and 35, VWR). Then, each portion was observed under a Contour GT-K1 Optical Profiler (Bruker Corporation, Billerica, MA) using the automated turret and programmable X, Y, Z movement. The diameter, area and surface roughness for each sample were measured in triplicate.

Bacterial culture. An avirulent, ampicilin-resistant and green fluorescent protein (GFP)-labeled *E. coli* O157:H7 strain B6914 (kindly provided by Dr. Pina Fratamico at the United States Department of Agriculture, Agricultural Research Service – Eastern Regional Research Center) was used. This strain was stored at -80 ℃ in tryptic soy broth (TSB; Becton Dickinson, Sparks, MD) with 20% glycerol.

Inoculation procedure. The frozen stock culture of *E. coli* O157: H7 strain B6914 was thawed and streaked on tryptic soy agar (TSA; Becton Dickinson, Sparks, MD) supplemented with 100 μg/ml of ampicilin (TSA-A), and grown at 37 ℃ for 24 h. Single colony of *E. coli* O157: H7 was streaked onto TSA-A again and incubated at 37 ℃ for 24 h. Colonies on the overnight plates were collected with sterile swabs and suspended in sterile saline (0.85% NaCl). The culture was centrifuged at 8,000 × rpm for 10 min, washed 3 times and resuspended in sterile saline (0.85% NaCl). The optical density at 600 nm (OD_{600}) of the culture was adjusted to ca. 0.7 to achieve a bacterial population of ca. 10^9 CFU ml^{-1}.

A 200 g portion of the finished compost sample with 20% moisture content was weighted in a sterile tray, and *E. coli* O157: H7 inoculum was sprayed onto the compost samples at a ratio of 1: 100 (vol: wt) with a sterile spray nozzle (sterilized with 70 % ethanol and rinsed with sterile tap water) at a final concentration of ca. 10^7 CFU g^{-1}. The compost samples were continually mixed by hands wearing sterile gloves (Sterile nitrile gloves, Kimberly-Clark, Neenah, WI) for 15 min under a biological hood. The mixed samples were then sieved into 3 portions: >1000, 500 -1000 and <500 μm using standard sieves.

Fluorescence microscopy observation of *E. coli* O157: H7. The finished compost sample inoculated with the GFP-labeled *E. coli* O157: H7 was observed under a fluorescence microscope, Nikon Eclipse Ti, 60X WI lens (Nikon instruments Inc., NY, USA). An uninoculated compost sample and GFP-labeled *E. coli* O157: H7 in broth were served as negative and positive controls, respectively. All of wide field mode, confocal mode and spectral mode visualization were performed using the 60 X objective with water immersion.

Spectral imaging and linear unmixing. Since GFP signal from *E. coli* O157: H7 and autofluorescence from compost were not separable under confocal mode, spectral analysis was attempted. The individual spectrum from GFP and compost autofluorescence was used for unmixing. Briefly, the same excitation light wavelength was applied to the samples inoculated with *E. coli* O157: H7 and the control, which is the finished compost sample without pathogen inoculation. Regions of interests representing emission spectra of GFP and autofluorescence were selected from the inoculated samples

and the control. Fluorescence signals were collected over the 400–700 nm spectral range at a spectral resolution of 5 nm/ channel. The emission wavelength spectrum of each region of interest was recorded as reference spectrum. These reference spectra were further used to differentiate sample signals.

Nikon's original imaging software NIS-Elements (Nikon Instruments Inc., Melville, NY) provides an integrated control of the microscope, cameras, components and peripherals. The image database, image acquisition and spectral analysis were also handled by the NIS-Elements imaging analysis software.

Immunofluorescence confocal microscopy. The antibodies were selected by their specific binding properties. The primary antibody, mouse GFP targeted antibody, was purchased from Invitrogen (Camarillo, CA). It is specific for the GFP protein, whereas, the second antibody is goat anti-mouse antibody conjugated with AlexaFluor 594 (Invitrogen), which has high affinity to the primary antibody and can produce a red signal with high intensity at a wavelength of 617 nm.

A ca. 0.5 cm^3 portion of the inoculated finished compost sample was added into a 2 ml centrifuge tube (VWR). Paraformaldehyde solution (16%, Electronmicroscopy Science) was diluted to 4% and filled into the 2 ml centrifuge tube. The compost sample in tube was fixed at 4 ℃ for overnight.

The fixed sample was centrifuged at 1,000 X rpm for 5 sec and the pellets were washed with 1X PBS containing 20 mM glycine (Sigma, St. Louis, MO). Then, the inoculated compost sample was blocked with above buffer for 10 min with rotation (Dynal Biotech, NY). After the tube was briefly centrifuged and supernatant was

removed, the sample was permeabilized with 0.05% Triton X-100 (Sigma, St. Louis, MO) for 20 min. Following washed by 1X PBS once, the sample was blocked with 1X PBS containing 3% bovine serum albumin (BSA) (w/v) and 10% goat serum for 20 min with rotation and then the sample was washed.

For IF staining, the primary GFP antibody was diluted in 1 X PBS containing 1% BSA (1: 20) (w/v). A few compost sample particles and the antibody solution were put into LabTek 8 well chamber (300 μl per well) and incubated at room temperature for 1 h. After the incubation, samples were washed with PBS containing 1% BSA twice. The second antibody was diluted in 1X PBS containing 1% BSA (1: 1000) (w/v), added into each sample chamber and incubated at room temperature for 2 h with rotation. Then the stained sample was washed twice with PBS/ 1% BSA and once with PBS. The specimens in each well were mounted in PBS/ glycerol (50: 50) (v/v) solution.

In order to minimize the background noise and nonspecific binding, several approaches were tested. First approach was to enhance the blocking with milk or trypan blue. After the samples were blocked with PBS containing 3% BSA (w/v) and 10% goat serum, additional blocking with reconstituted milk (concentration) or trypan blue was applied. The second approach was to optimize antibody concentration and reaction time. The primary antibody concentration was changed from 1:20 to 1:50. Separately, the effect of changing the 2^{nd} antibody reaction time from 2 to 1 h was tested.

Microscopic observation of compost samples labeled with Qdot® secondary antibody conjugates. The finished compost samples with particle sizes of 500-1000 and <500 μm were prepared and inoculated as described above. The primary antibody, mouse

GFP targeted antibody, was the same used in IF protocol. The secondary antibody conjugate used in this study was Qdot® 585 Goat F(ab')2 Anti-Mouse IgG Conjugate (H+L) (emission wavelength 585 nm) (Invitrogen, Camarillo, CA). They were prepared as described in the manufacturer guide. Briefly, the Qdot® secondary antibody conjugate vial was centrifuged at 5,000 X g for 3 min and the pellets, which are the aggregation of Qdots, were discarded. The supernatant was diluted with PBS containing 3% BSA (w/v) in a ratio of 1: 25 to yield a final concentration of 40 nM prior to use.

The compost samples were fixed and quenched as described above in IF protocol. The fixed compost samples were washed 3 times for 5 min with 1X PBS, followed by permeabilization for 20 min in 0.05% triton X-100 (VWR) in PBS. Then the samples were aspirated and the blocking buffer (6% BSA+10% goat serum in PBS) was added. After 1 h of incubation at room temperature, 1st antibody was diluted in PBS (1:20) and incubated for another 1 h, followed by washing 3 times with 1X PBS for 5 min each. The diluted Qdot® secondary antibody conjugate was added and incubated at room temperature for another 1 h, followed by another washing for 3 times. Then, the samples were mounted in PBS and placed into LabTek 8 well chamber. Samples were observed under the Leica SP8X multiphoton with confocal system (Leica, Mannheim, Germany).

Results

Morphological and surface analysis of compost particles by surface profiler. All the compost samples with different particle sizes were measured in triplicate using the Contour GT-K1 Optical Profiler. The samples with particle sizes of >1000, 500-1000

and <500 μm had an average diameter of 2,037, 884 and 257 μm, respectively. The average surface roughness (Ra) were 112.66, 76.61 and 26.45 μm for samples with particle sizes of > 1000, 500-1000 and <500 μm, respectively. Ra is defined as the arithmetic average of the absolute values of height difference. The average areas were 1.19×10^6, 2.91×10^5 and 3.70×10^4 μm^2 for particle sizes of >1000, 500-1000 and <500 μm, respectively. Data of the profiler analysis were summarized in Table 3.1, whereas images of those tested particles were presented in Fig 3.1- Fig 3.3.

Fluorescence microscopy observation of *E. coli* O157: H7 in compost. Both pure culture of GFP-labeled *E. coli* O157: H7 and inoculated compost (ca. 10^7 CFU g^{-1}) were observed under fluorescence microscope (Fig 3.4; 3.5). As a matrix control, uninoculated finished compost sample was observed in wide field of the microscope (Fig 3.3). The number and intensity of green fluorescence signal were much higher in *E. coli* O157: H7 pure culture than those in the inoculated compost sample. This suggests that compost materials significantly reduce the sensitivity of the observation of green fluorescence proteins from *E. coli* O157: H7.

Spectral analysis and linear unmixing. To separate the GFP signal from *E. coli* O157: H7 and the autofluorescence from compost matrix, spectra analysis and linear unmixing were attempted. The spectral analysis data are shown in Fig 3.6. The highest emission intensity was observed at wavelength of ca. 500 nm for the GFP in *E. coli* O157: H7 cells, while the maximum emission intensity of the background autofluorescence was below 480 nm according to the spectra data. The maximum intensity of GFP at wavelength of 500 nm was significantly higher than the maximum

intensity of autofluorescence at 500 nm. Since the background autofluorescence intensity is high, the emission of GFP is still not easily observed in microscope.

Immunofluorescence confocal microscopy observation of compost samples. When the IF protocol was used, the GFP in *E. coli* O157: H7 was stained as red while the compost autofluorescence remained green. For the IF protocol described in Materials and Methods, GFP in *E. coli* O157: H7 and autofluorescence of compost can be differentiated both visually and quantitatively according to the signal intensity. The images of the IF of thin and thick samples are shown in Fig 3.7 and 3.8, respectively. By using the IF method, the AlexaFluor-594 stained GFP-labeled *E. coli* O157: H7 cells can be visualized in the compost matrix with low noise (no significant red dots in the control). In the thin samples, the light was able to pass through samples which resulted in clear background and obvious red IF stained cells (arrows in fig 3.7). However, in the thick samples the images were dark and the target red signal is not as clear as it is in thin samples. The intensity profile shows that the AlexaFluor-594 stained GFP has significant higher intensity compared to the control compost sample in the red channel. The maximum intensity of the compost sample inoculated with GFP-labeled *E. coli* was 1500 (Fig 3.9), whereas the maximum intensity of the control sample was 440 (Fig 3.10). However, among all the compost samples, the GFP and the autofluorescence can be differentiated in only a portion of samples since the control sample also has red emission signals (Fig 3.10).

In an attempt to improve above IF method, several modifications of the method were tried, including enhancing the blocking with milk or trypan blue and optimizing

antibody concentration and reaction time. However, the results didn't show much improvement of GFP-labeled *E. coli* O157: H7 detection. The target emission light was not separable from the autofluorescence.

Microscopic observation of samples stained with Qdot® secondary antibody conjugates. The compost samples inoculated with GFP-labeled *E. coli* O157: H7 with Qdots were observed under the Leica multiphoton with confocal system, which have higher sensitivity of emission signals. The *E. coli* O157: H7 cells were viewed as rod structures with green and red overlap emission signals in compost samples under the microscope. The microscopic images of samples with <500 μm particle size revealed that the inoculated sample had *E. coli* O157 cells in compost matrix while the control sample had no obvious target signals (Fig 3.11 A&B). However, the control samples still had high intensity of autofluorescence even subtracted by NIS-Elements imaging analysis software (Fig 3.11 C&D). Based on the images, it is clearly to say that GFP in *E. coli* O157: H7 stained with Qdots was brighter in compost matrix than it in AlexaFluor-based IF methods. However, for the samples with particle size of 50-1000 μm, Qdots conjugates IF images revealed E. coli O157: H7 cells at the edge of compost particles but not in the center (Fig 3.12). Therefore, the difference of pathogenic cell interaction with compost particle cannot be compared with current microscopic resolutions.

Discussion

The compost samples with different particle sizes were analyzed under a surface profiler to determine the diameter, area and surface roughness. The results for sample

particle sizes are in good agreement with the sieve pore sizes which were 500 and 1000 μm (Table 3.1). In compost particles, the surface roughness (Ra) represents the space where pathogens shelter themselves. Since pathogens can attach to particle surface, the complex surface structure can prevent pathogenic cells from being washed away by wind or water. In this study, the Ra results suggested that large particles had larger average surface area per particle than small particles (Table 3.1). In fact, under microscope, the large particle had more complex structure while the small particles tend to have smooth surface (Fig 3.1-3.2). For example, sand particles usually exist in compost and most of them are under 500 μm and have smooth surface. All these factors contributed to the conclusion that large compost particles tend to have better abilities to hold and protect pathogenic cells in them, which supported our previous results on better survival of *E. coli* O157: H7 and *Salmonella* in large particles (Chapter 2, Table 2.1 and 2.2).

In this study, we used epifluorescence microscopy to directly observe GFP-labeled *E. coli* O157: H7 in compost matrix. And the results showed that it is not a good method in analyzing compost samples due to the interference from autofluorescence of compost matrix. However, some studies have shown successful observation of bacteria in soil using epifluorescence microscopy directly. Pascaud et al. (2008) used epifluorescence microscopy to observe viable cells in soil. To increase the contrast sensitivity, they stained the cells using LIVE/DEAD method. Abbey et al. (2003) successfully visualized GFP-labeled *Ralstonia eutropha* H850 under epifluorescence microscope in soil. The GFP was chromosomally labeled in above organism and this makes the green fluorescence more stable. However, in our study, the GFP was encoded

on a plasmid, which may cause unstable GFP expression due to the growth of microorganisms.

To try to reduce background signal interference, spectral analysis and linear spectra unmixing was used in this study. The emission spectra of GFP from *E. coli* O157: H7 and autofluorescence were differentiated by linear unmixing. Anderson et al. (2006) used linear unmixing method to separate GFP and red FP variants on Zeiss LSM 510 in their experimental set-up. The 594 nm HeNe was selected to be the red fluorophore excitation source, while the 488 nm was used as GFP excitation source. This configuration was designed to maximize sensitivity and minimize crosstalk. Baird et al. (2012) used 5 different fluorophores to label DNA, protein, lipids and two types of extracellular polysaccharides on a *Pseudomonas aeruginosa* biofilm. Standard channel mode and spectral imaging were used to image the biofilm sections. With the careful selection of multiple fluorophores, optimization of the staining protocol, use of appropriate controls, and utilization of spectral imaging with linear unmixing, they were able to have reproducible and reliable images of biofilm. However, the linear unmixing technique is limited by factors such as the image background level, noise, and the relationship of the emission peaks to the detection channels when imaging living cell specimens (Anderson et al., 2006).

In this study, using the linear unmixing method, the emission intensity of GFP at ca. 500 nm was higher than that of the compost. However, the method failed to provide images that can clearly show the location of *E. coli* O157: H7 in compost samples due to the broad spectrum and high intensity of the background autofluorescence of the compost

sample (Fig 3.6). Thus, the IF staining method was investigated for its ability to enhance the target fluorescence signal intensity and differentiate target signal from autofluorescence by changing the target signal emission color. When the signal of *E. coli* O157: H7 was changed to red by using antibody coupled with red fluorescence dye AlexaFluor 594, the broad spectrum autofluorescence in blue-green zone from the compost did not affect the visualization of *E. coli* O157: H7 as much as directly observing GFP (Fig 3.7). Urakawa et al. (2006) used IF staining method to label the beta-proteobacterium in ammonia-oxidizing bacteria (AOB). They reported that good fluorescence signals were obtained from both β and γ-AOB strains. Ammar et al. (2005) used IF staining technique to study distribution of the bacterium *S. kunkelii* in vector leafhoppers and their results demonstrated that the IF technique was effective to label proteins in tissue matrix. Welter et al. (2002) also were successful to use IF protocol to label proteins in microorganisms. However, the IF staining microscopy technique is limited by photobleaching. The fluorescence antibody in the stained sample tends to be destroyed by long-time exposure to excitation light, which can be prevented by reducing the intensity or time-span of light exposure. IF staining method has its advantage of processing the sample considerably fast, able to label target with intended wavelength of fluorescence antibody. However, the high autofluorescence intensity still significantly affects the observation of cells labeled with fluorescence antibodies. Additionally, the number of cells can be seen in the compost samples are limited, which may result from non-specific binding to compost components.

To enhance the signal of target, quantum dots conjugates were used to detect GFP signal in this study. The Qdots are produced strictly uniform in size, and the emission light wavelength are associated with their specific sizes. Thus, the emission spectra of quantum dots conjugates are narrow and symmetric. Unlike IF staining, the Qdots are much brighter and photostable. In this study, the images showed brighter signals and more obtainable cells when the Qdots concentration is 40 nM (Fig 3.11) than the control in both <500 and 500-1000 μm samples. The Qdots staining was able to detect more cells under the microscope compared with IF staining method. Nevertheless, the compost background still had strong autofluorescence. This may also result from some non-specific binding of compost material or indigenous microorganisms with Qdots. Besides, the Qdots stained compost samples were observed in the Leica confocal microscope which is more sensitive about emission signal than the Nikon Eclipse Ti system. This may enlarge target emission signal as well as the autofluorescence signal. We have tried to compare the difference in interaction of *E. coli* O157: H7 cells with different compost particle sizes, however, the quality of images for large and thick compost samples was not sufficient to make a meaningful conclusion. In summary, Qdots labeling method has its advantage of brighter emission light and narrow spectra, but the strong autofluorescence is the major drawback.

The key to visualize target bacterial cells in complex matrix is to increase the signal to noise ratio. Beside IF and Qdots stain methods, the fluorescence in situ hybridization (FISH) method has been mentioned by other researchers and could be a promising approach of our future study. The FISH method is able to target on a specific

DNA sequences on chromosomes and bind them. As compared to the IF staining method, FISH has the advantage that it minimizes the non-specific binding, so the background noise is decreased. FISH method provides rapid turnaround time, increased sensitivity and information on the structure of the microbial community as compared to conventional culture method and PCR methods (Whiley et al., 2011). Unfortunately, when FISH applied to soil, the specificity of probes is reduced due to greatly aggregated cells, nonspecific binding (Christensen et al., 1999) and autofluorescence in soil (Whiley et al., 2011). They also suggested that the drawbacks can be reduced when applied together with other techniques. Whiley et al. (2011) stated that spectral unmixing was the best microscopy technique for reducing background fluorescence when using FISH method. Although the use of the method in soil is still debatable, considering the complex nature and abundant indigenous microflora in soil and compost matrix, the more specific method such as FISH may provide better visualization results, which needs to be evaluated in the future.

This study explored several microscope methods for visualizing *E. coli* O157: H7 in compost. Although GFP is a good marker for microbial cell imaging, the interference of compost autofluorescence makes the visualization of cells very difficulty even with the aid of spectral unmixing. However, the visualization of fluorescence labeled cells in compost matrix can be achieved by immunofluorescence staining techniques, such as AlexaFluor 594 and quantum dots. How to minimize the strong background autofluorescence as noise needs to be investigated further.

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Figure Legends

Fig 3.1 3D Optical Profiling of Compost Samples with >1000 μm Particle sizes. Particles observed were randomly selected in the sieved compost sample. Color on the sample represents the height of the particle. Red represents high part while blue represents low part.

Fig 3.2 3D Optical Profiling of Compost Samples with 500-1000 μm Particle sizes. Particles observed were randomly selected in the sieved compost sample. Color on the sample represents the height of the particle.

Fig 3.3 3D Optical Profiling of Compost Samples with <500 μm Particle sizes. Particles observed were randomly selected in the sieved compost sample. Color on the sample represents the height of the particle.

Fig 3.4 finished compost samples in wide field mode. The compost sample with 500-1000 μm particle size was observed under wide field microscope.

Fig 3.5 GFP labeled *E. coli* O157 cell suspension under fluorescence microscope. The *E. coli* O157 suspension concentration was 10^7 CFU/ml. And the objective was 10X.

Fig 3.6 finished compost sample inoculated with *E. coli* O157: H7. The *E. coli* O157 population in compost sample was 10^7 CFU/g. The compost sample with 500-1000 μm particle size was observed under fluorescence microscope. And the objective was 10X.

Fig 3.7 Spectral analysis of GFP labeled *E. coli* O157: H7 in compost. The wavelength ranged tested was 460-640 nm, and the interval was 5 nm.

Fig 3.8 IF images for GFP labeled *E. coli* O157: H7 in compost (thin samples). The compost sample with 500-1000 μm particle size was labeled and observed. From the 500-

1000 μm samples, thin particles which allow light pass through. The above image is control sample and the bottom image is from inoculated sample.

Fig 3.9 IF images for GFP labeled *E. coli* O157: H7 in compost (confocal thick samples). Thick samples from 500-1000 μm particle size portion were selected to observe. The above image is control sample and the bottom image is from inoculated sample.

Fig 3.10 IF images for GFP labeled *E. coli* O157: H7 in compost in red channel. Samples inoculated with GFP-labeled E. coli O157 were observed. The emission intensity in a line (labeled blue) was recorded and put into graph. The maximum intensity was 1500.

Fig 3.11 IF images for compost (control) in red channel. Compost sample without inoculation was observed. The emission intensity in a line (labeled blue) was recorded and put into graph. The maximum intensity was 440.

Fig 3.12 3D images of E. coli in compost sample labeled with Qdots (small samples). Image of control sample without inoculation (above) and inoculated compost sample (bottom) were observed. The green and red overlap in these images indicating that the green rods are target *E. coli*.

Fig 3.13 Background subtracted images of *E. coli* in compost sample labeled with Qdots (Small samples). Image of control sample without inoculation (above) and inoculated compost sample (bottom) were observed. The green and red overlap in these images indicating that the green rods are target *E. coli*. The background fluorescence was subtracted partially by Nikon software.

Table 3.1 The 3D profile analysis of compost samples with different particle sizes

Sample size	Sample No.	Ra (μm)*	Area (μm^2)**	Diameter (μm)***	Volume (μm^3)
>1000 μm	1	142.80	1.35×10^6	1965	7.35×10^8
	2	77.79	1.27×10^6	2022	5.98×10^8
	3	117.38	9.60×10^5	2123	4.24×10^8
	average	112.66±32.76	$1.19\times10^6\pm2.04\times10^5$	2037±80	$5.85\times10^8\pm1.56\times10^8$
500-1000 μm	1	63.99	2.21×10^5	853	4.33×10^7
	2	72.12	3.03×10^5	864	7.09×10^7
	3	93.73	3.49×10^5	934	1.15×10^8
	average	76.61±15.37	$2.91\times10^5\pm6.48\times10^4$	884±44	$7.63\times10^7\pm3.60\times10^7$
<500 μm	1	19.44	3.20×10^4	267	2.26×10^6
	2	14.70	8.00×10^3	122	4.60×10^5
	3	45.20	7.10×10^4	382	1.04×10^7
	average	26.45±16.41	$3.70\times10^4\pm3.18\times10^4$	257±130	$4.37\times10^6\pm5.29\times10^6$

*Ra, represents the surface roughness. It is the arithmetic average of the absolute values of height difference.
**Area and volume represent the measurement per particle.
**Diameter is the average measurement of particle length.

Fig 3.1 The 3D optical profiling compost samples with >1000 µm particle sizes

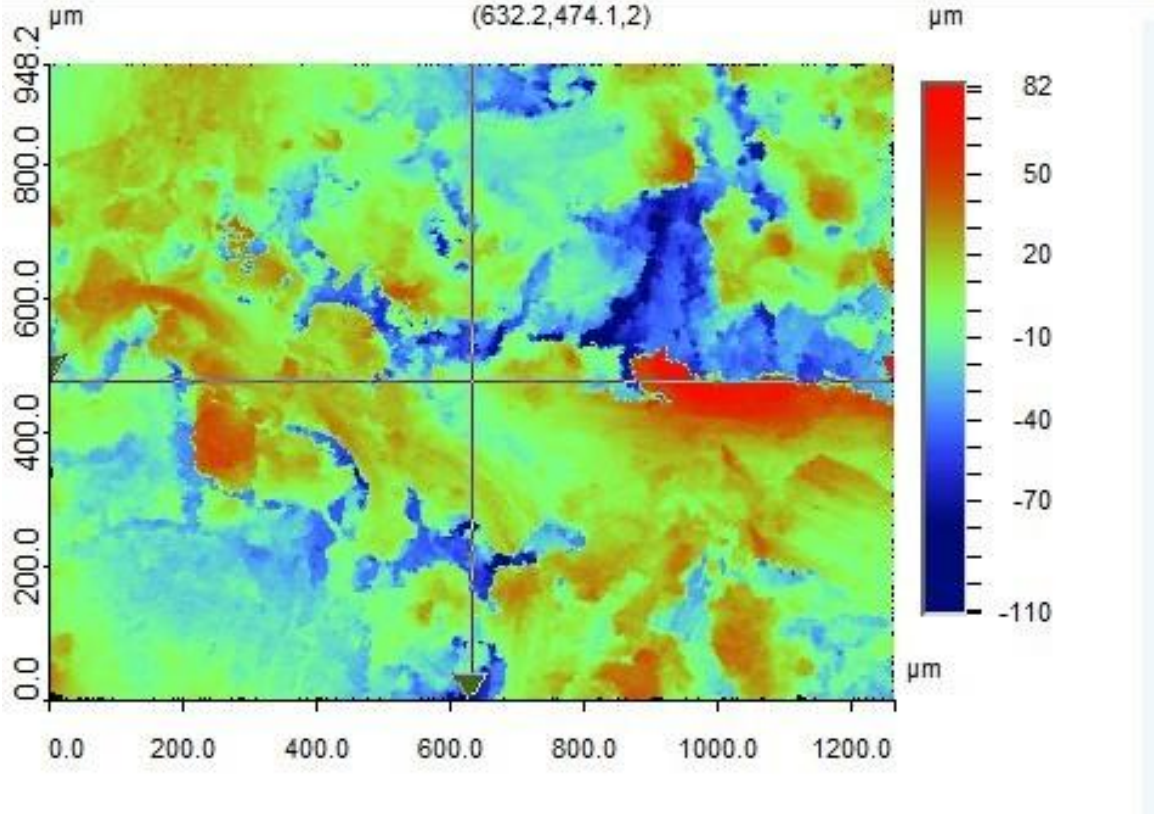


Fig 3.2 The 3D optical profiling of compost samples with 500-1000 μm particle sizes

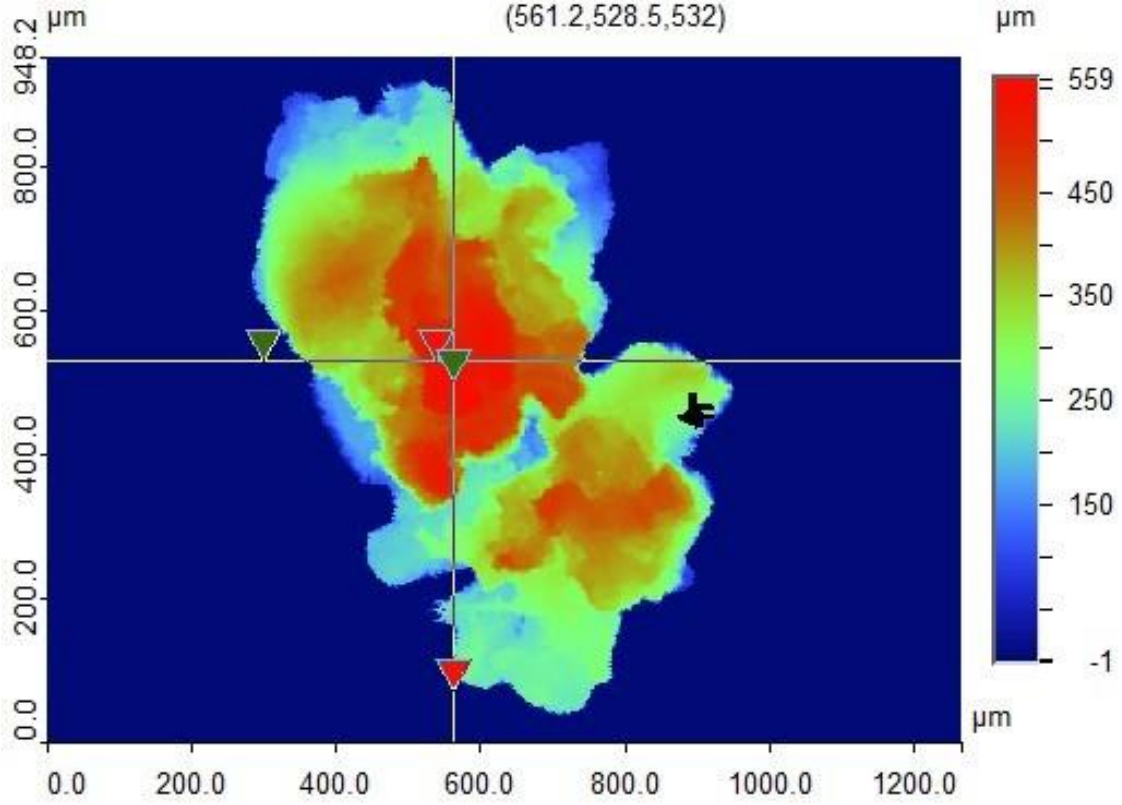


Fig 3.3 The finished compost samples observed in wide field mode

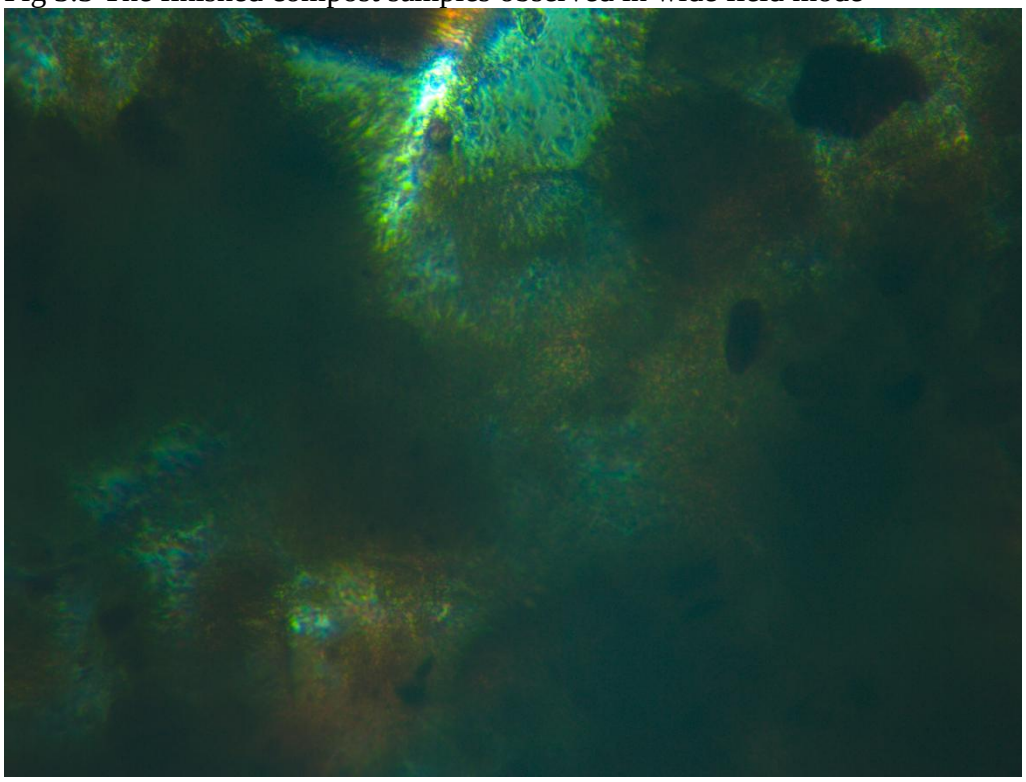


Fig 3.4 The GFP-labeled *E. coli* O157: H7 cell suspension under fluorescence microscope

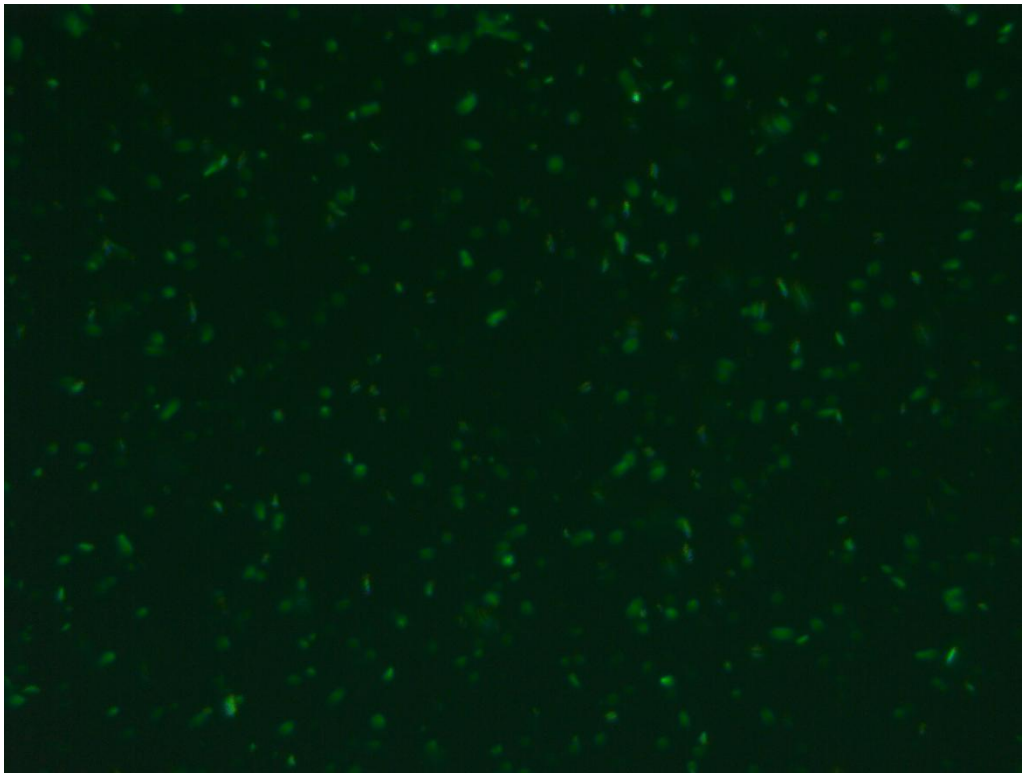


Fig 3.5 The finished compost sample inoculated with GFP-labeled *E. coli* O157: H7

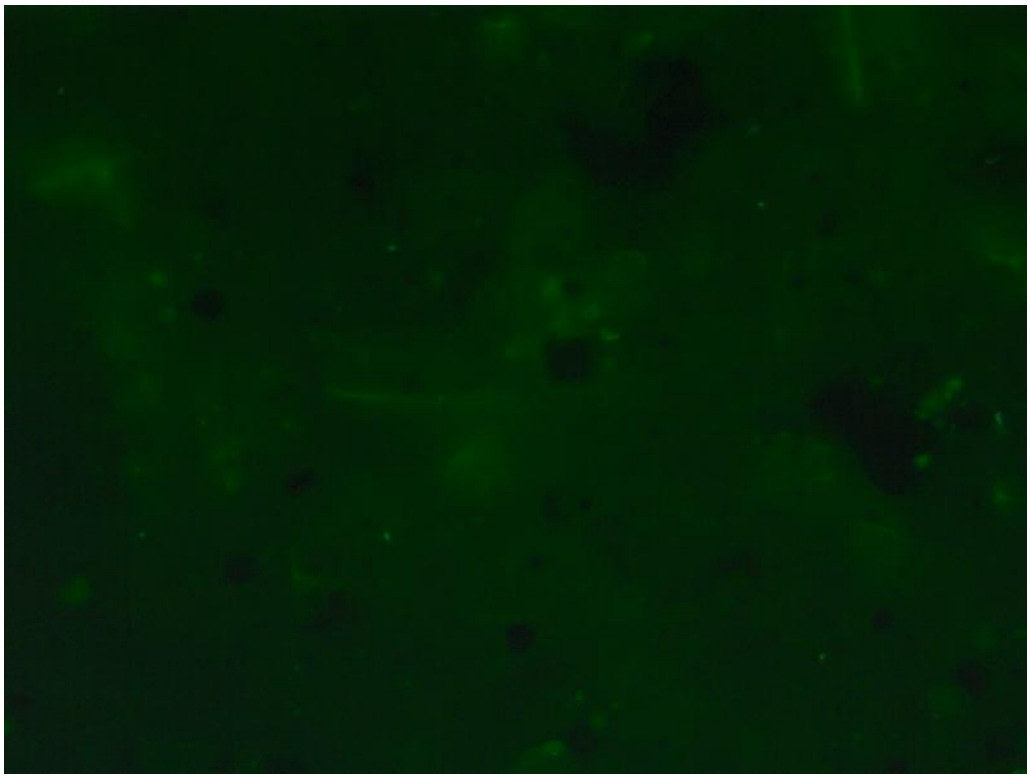


Fig 3.6 Spectral analysis of GFP-labeled *E. coli* O157: H7 in compost

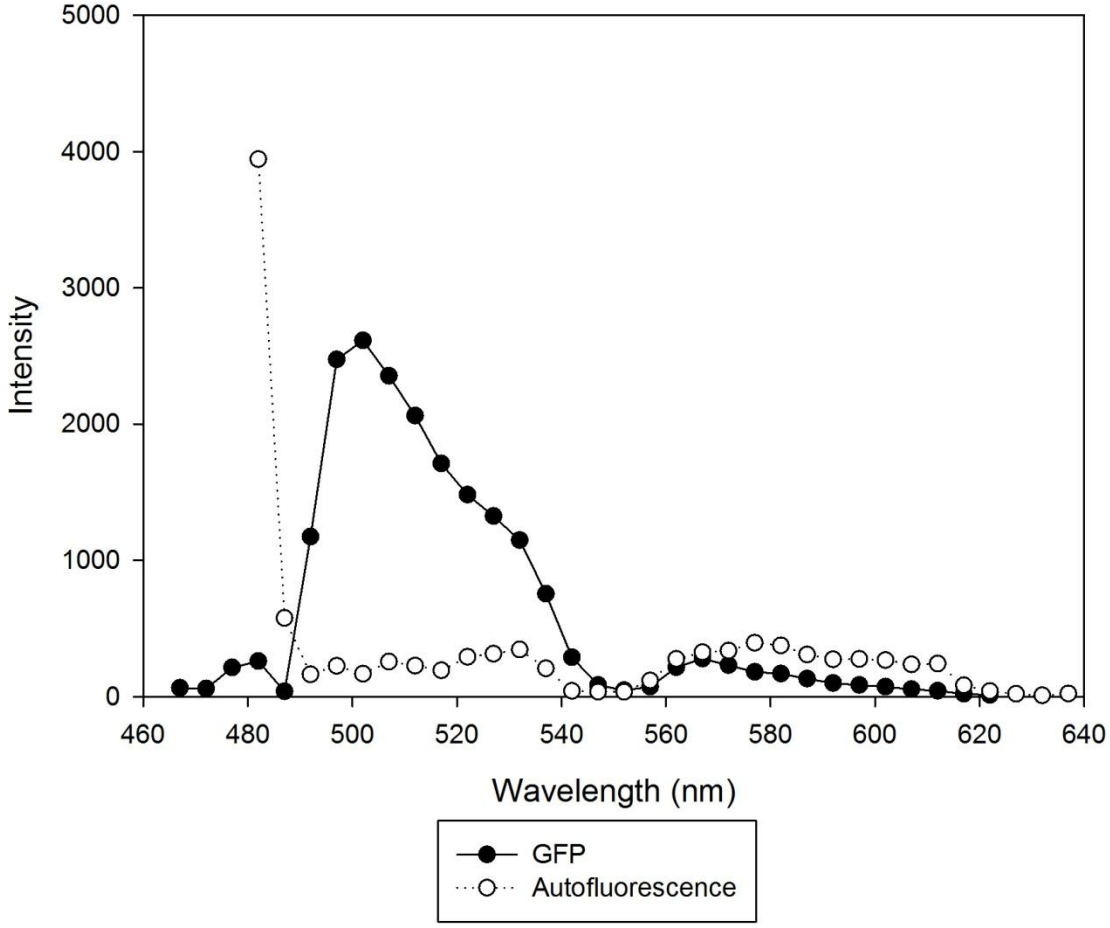


Fig 3.7 IF images of GFP-labeled *E. coli* O157: H7 in compost (A, thin samples) and compost control (B)

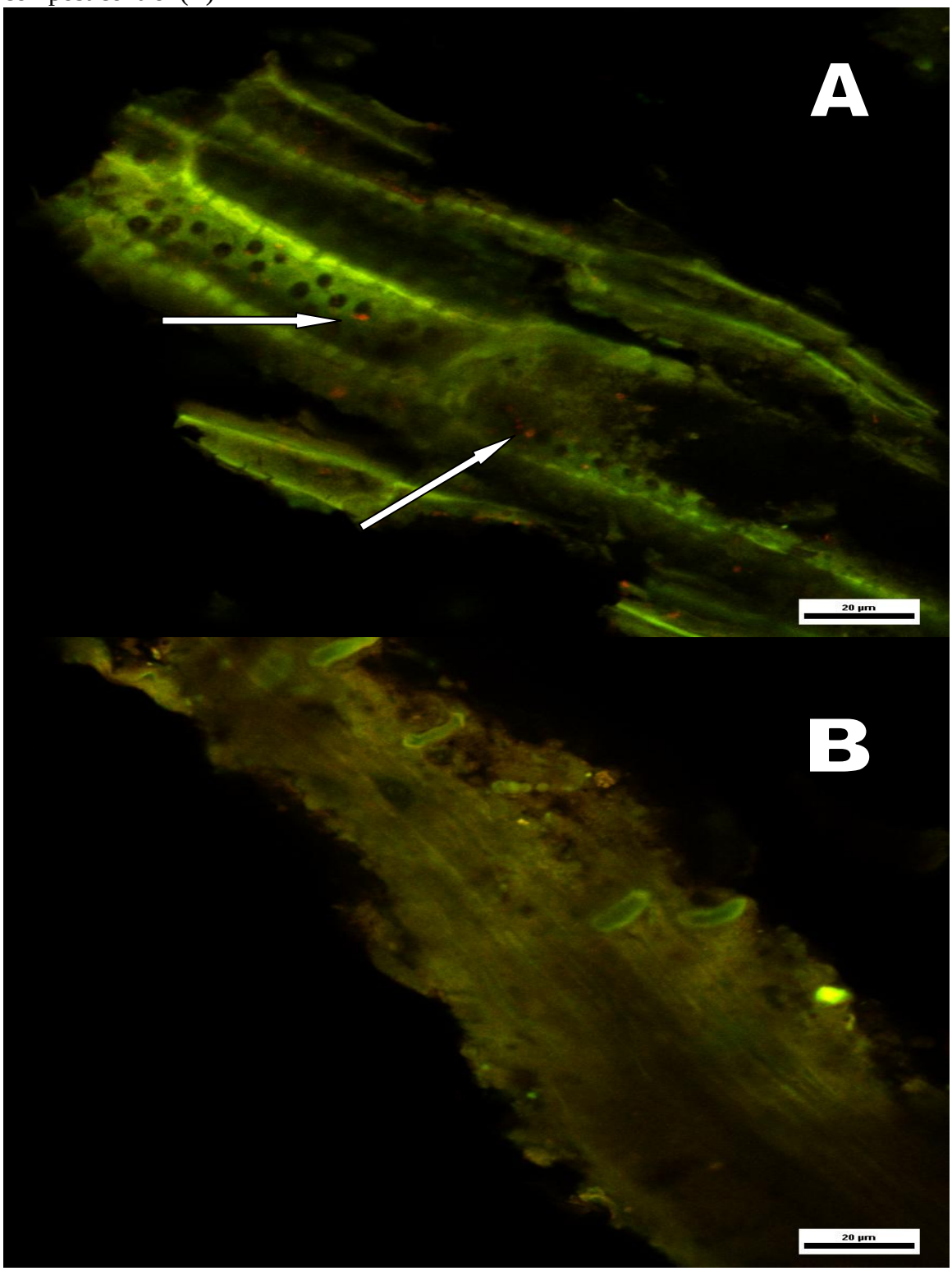


Fig 3.8 IF images for GFP-labeled *E. coli* O157: H7 in compost (A, confocal thick samples) and compost control (B)

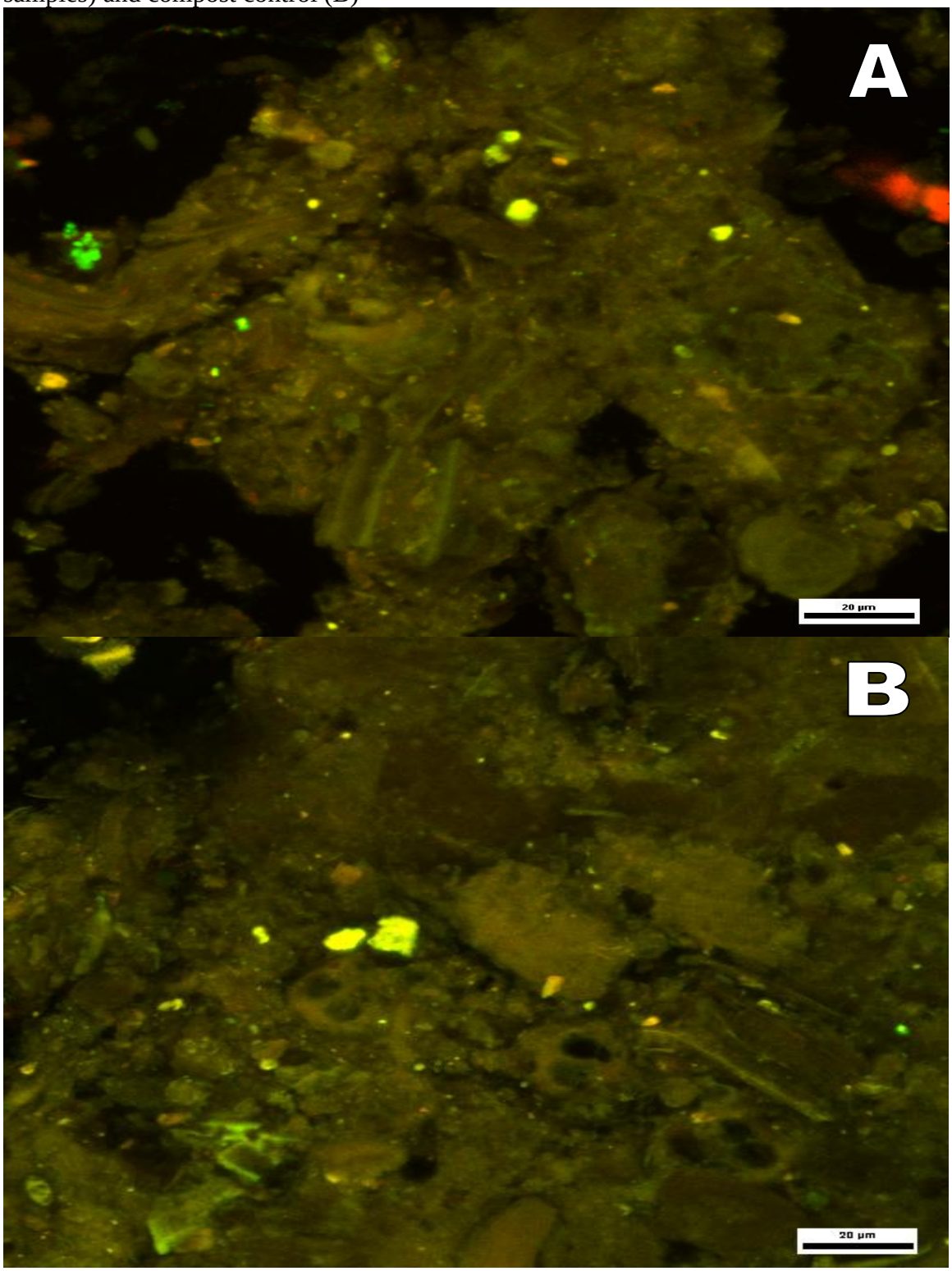


Fig 3.9 IF images for GFP-labeled *E. coli* O157: H7 in compost in red channel

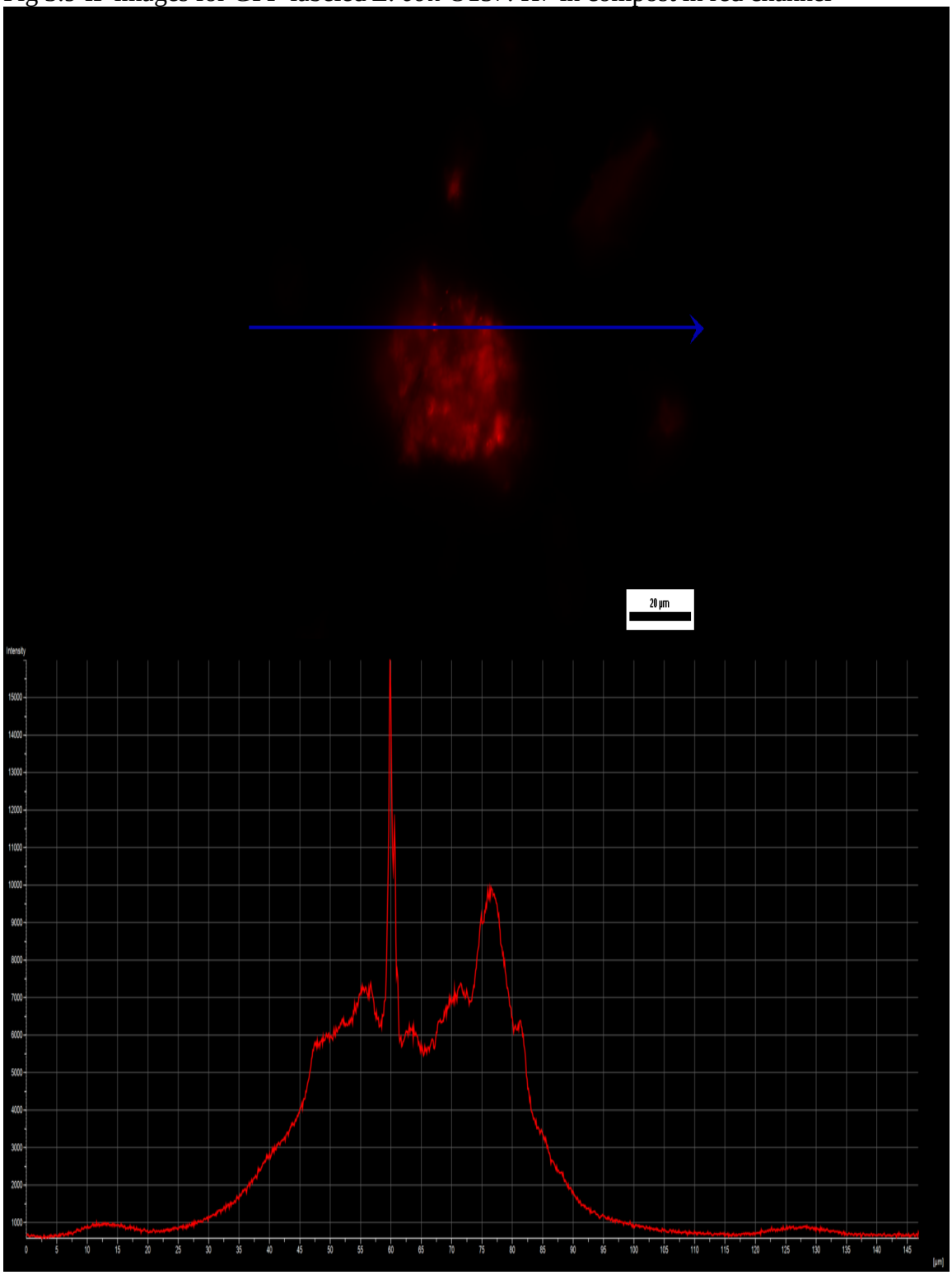


Fig 3.10 IF images for compost (control) in red channel

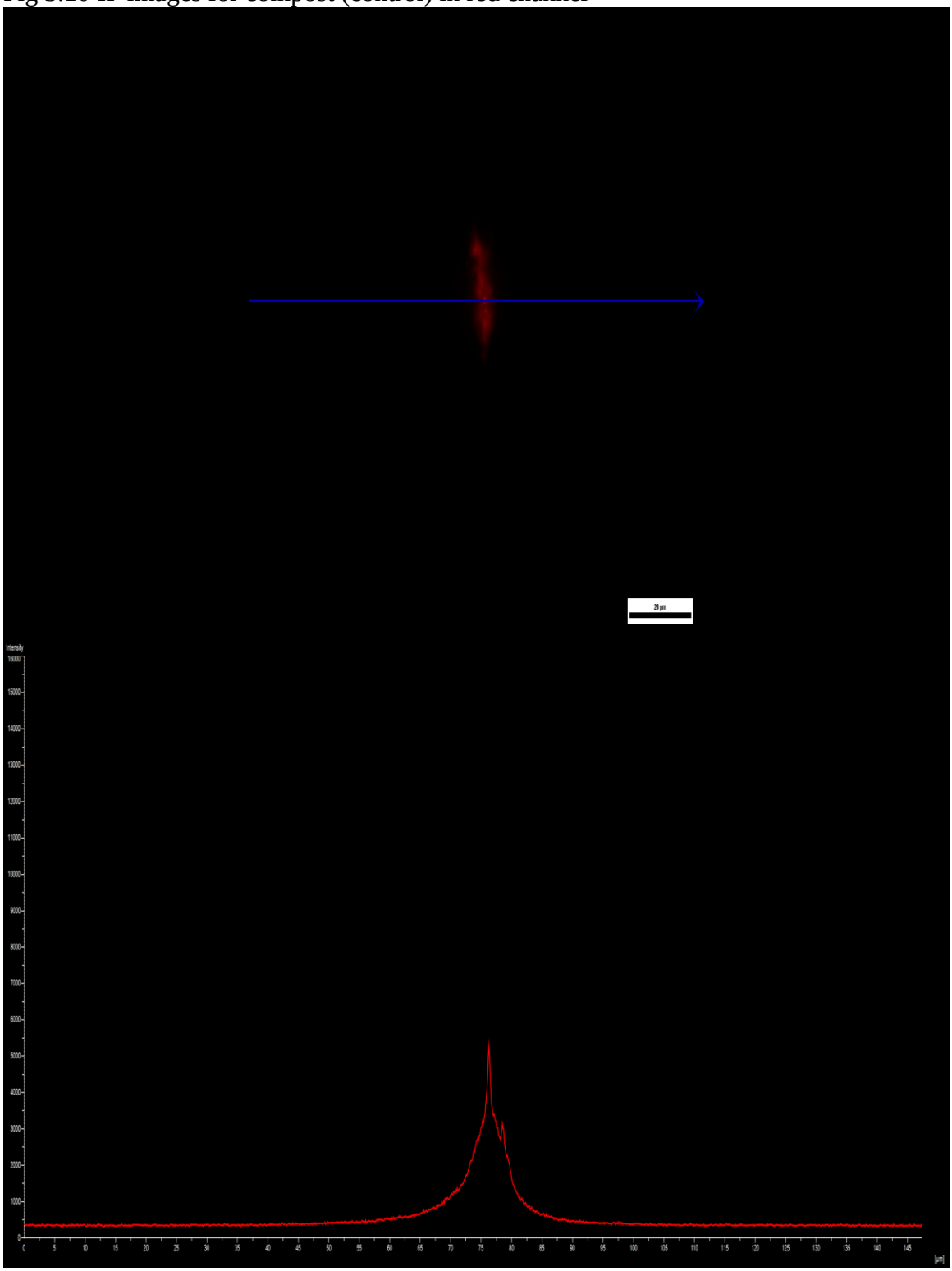


Fig 3.11 3D images of GFP-labeled *E. coli* O157: H7 in compost sample with small particle size detected by Qdots (A) and control (B). And background subtracted image (C) and control (D).

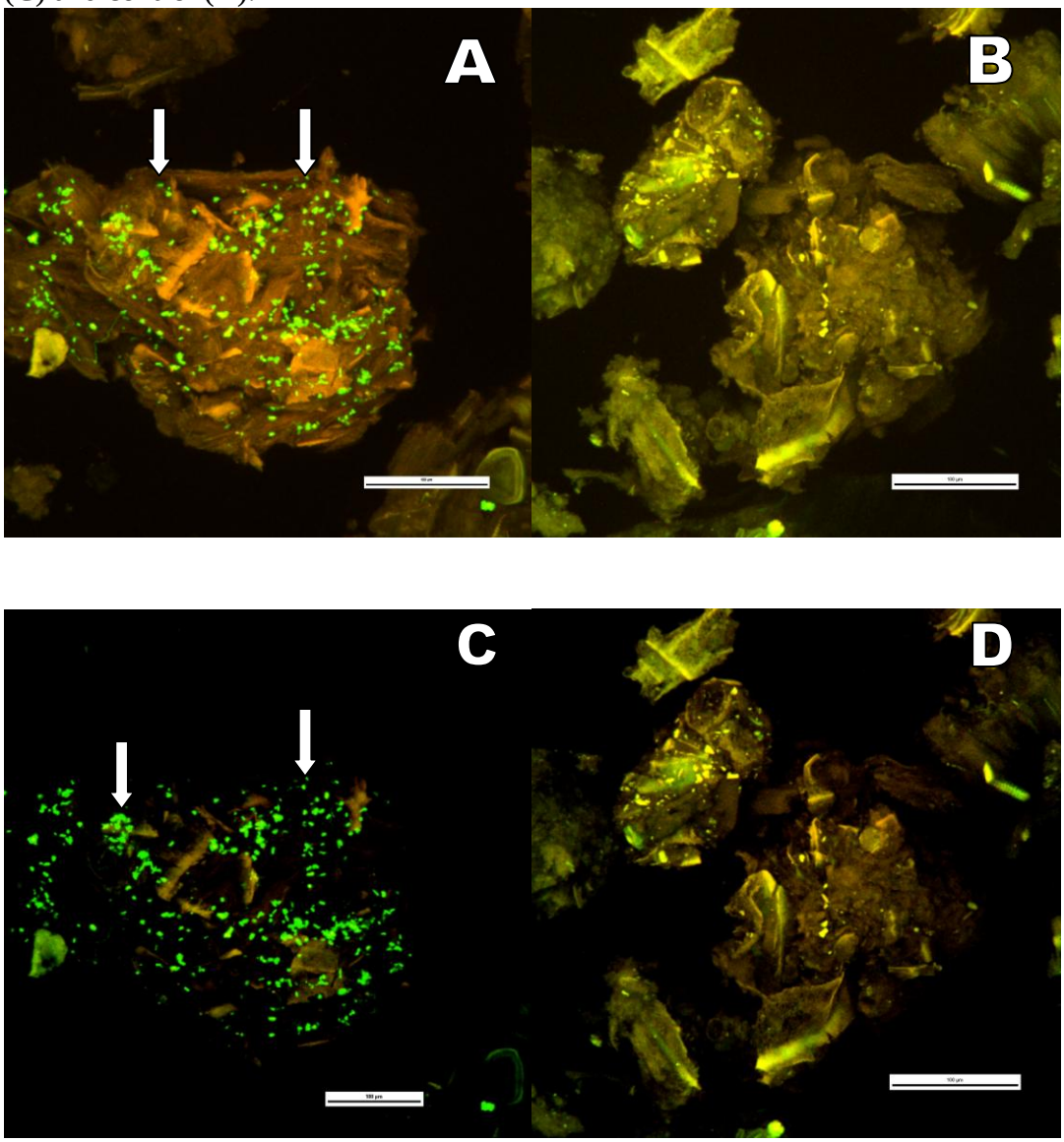
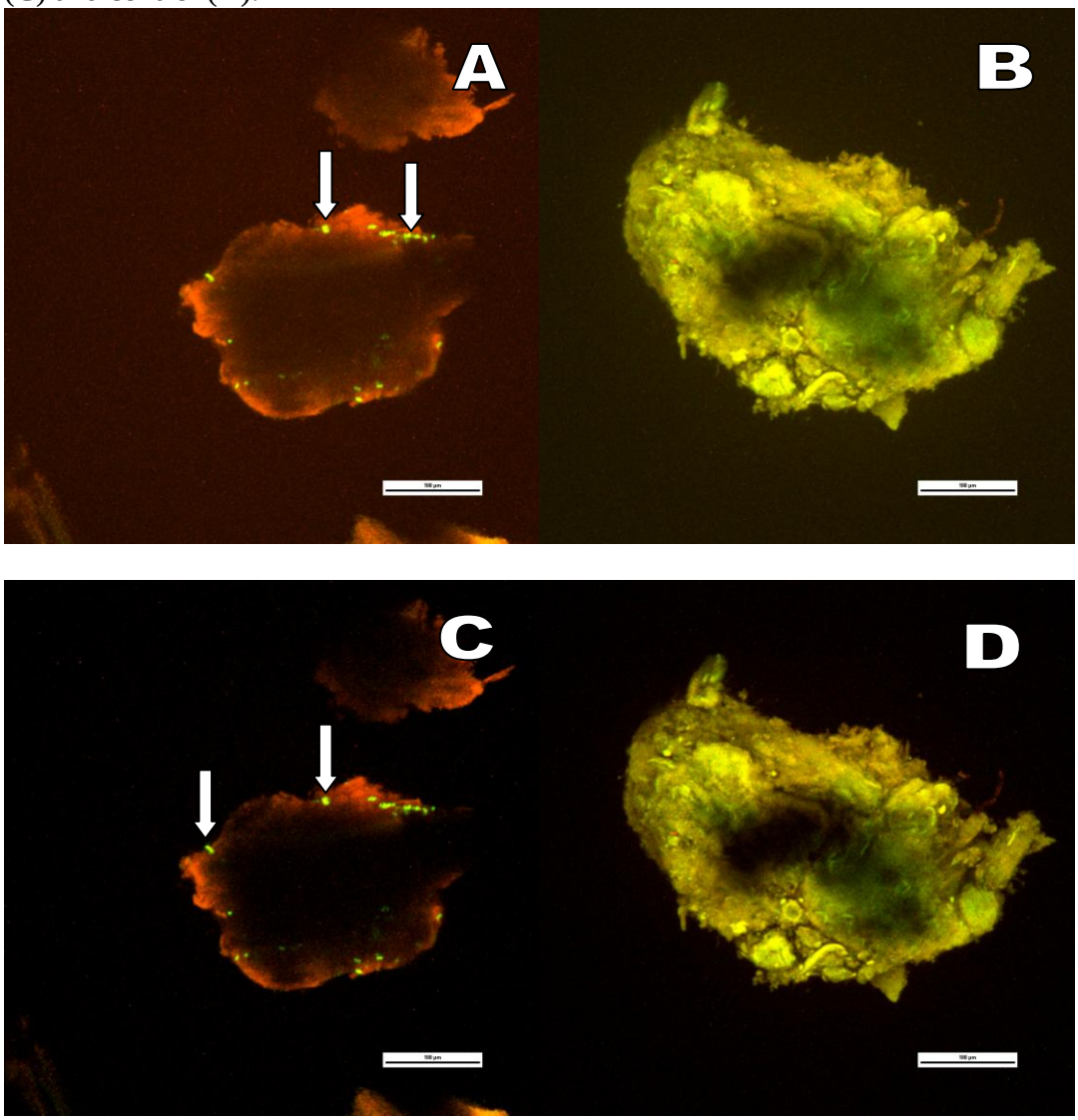


Fig 3.12 3D images of GFP-labeled *E. coli* O157: H7 in compost sample with medium particle size detected by Qdots (A) and control (B). And background subtracted image (C) and control (D).



CHAPTER FOUR

CONCLUSION

In this study, pathogen survival in finished compost was conducted under greenhouse conditions. The results showed that higher dehydration rate was a critical factor which contributes to the rapid inactivation of pathogens, and compost samples with larger particle sizes support pathogen survival for extended time as compared with compost samples with smaller particle sizes. Also, microscope methods were explored to observe GFP-labeled *E. coli* O157: H7 in compost matrix and the results showed that amplification of GFP signal by immunofluorescence methods, esp. Qdots, can improve the detection of target cells. However, these microscope methods need to be optimized in order to understand the interaction of pathogen in complex compost matrix.

Based on our findings, we'd suggest that the composting facilities should be kept away from fresh produce field, as the transmission of larger compost particles with higher possibilities of foodborne pathogen survival via air happens when the composting facility and the fresh-produce field are close enough. When they are far away, even the smaller compost particles may be transmitted to fresh-produce field via bioaerosols, although less pathogen survival on smaller compost particles was observed in this study. However, to accurately assess the risk of composting facilities as a source of bioaerosol, further studies are needed to investigate the ability and risk of the transmission of compost with different particle sizes in open environments.