THE ROLE OF HOUSE FLIES IN THE ECOLOGY OF ENTEROCOCCI FROM
WASTEWATER TREATMENT FACILITIES

by

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B.S., Central Missouri State University, 1996
M.S., Oklahoma State University, 1999

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

Enterococci are a group of commensal bacteria that are important nosocomial pathogens. They are abundant in human sewage and wastewater treatment facilities (WWTF). This study focused on the role of house flies, *Musca domestica*, in the ecology of enterococci at WWTF in both field and laboratory experiments. The first study objective focused on sampling and characterizing enterococci from house flies and wastewater sludge from four WWTF in northeastern Kansas. Enterococci were quantified, identified, and screened for antibiotic resistance and virulence traits, and genotyped. The profiles of enterococci (spp. diversity, antibiotic resistance and virulence) from WWTF sludge and the house flies were similar, indicating that the flies successfully acquired the bacteria from the WWTF substrate. Enterococci with the greatest amount of antibiotic resistant and virulence traits originated from the WWTF that processed meat waste from a commercial sausage plant. Genotyping of *E. faecalis* revealed clonal matches from sludge and house flies. The second study objective involved tracking the fate of *E. faecalis* in the digestive tract of house flies in laboratory assays. Colony forming unit (CFU) counts were highest in the midgut at 1 h and declined during the first 24 h. In the labellum, foregut and hindgut, *E. faecalis* concentrations were more variable but were overall higher after 24 h. Observations from CFU counts and visualizations under a dissecting microscope revealed that *E. faecalis* peaked in the crop after 48 h suggesting active proliferation in this region. The third objective of the study involved tracking the emergence of calyptrate muscoid flies from stockpiled biosolid cake at one of the four WWTF. Traps were employed at the site for a total of 47 weeks, totaling 386 trap-weeks. A total of 11,349 calyptrate muscoid flies were identified with the two most common species being stable flies

(*Stomoxys calcitrans*) (9,016, 80.2%) and house flies (2022, 18.0%).   Numbers of stable flies and house flies peaked around mid-July each year and a second, smaller peak was observed for stable flies 5-8 weeks later.   Estimated annual emergence of stable flies was 551,404 and for house flies 109,188.

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CHAPTER 1: LITERATURE REVIEW, RESEARCH GOAL AND OBJECTIVES

1.1 THE HOUSE FLY (*MUSCA DOMESTICA* (L.)) AND ITS ROLE IN DISEASE TRANSMISSION

The house fly, *Musca domestica* (L.) is the most common fly species in the family Muscidae and is distributed worldwide. The adult fly measures 8-12 mm in length, coloration is grey with a pale ventral abdomen and the dorsal thorax is characterized by four dark grey longitudinal stripes. Development is holometabolous with life stages of egg, larvae, pupae and adult. Upon emergence from the egg, the fly larva develops through three instars in about 7-9 days. Mature 3rd instar larvae seek a dry, concealed area to pupate. The pupal stage lasts for 5 days, adults emerge from the puparium and live 2-3 weeks (Kettle 1995).   Females mate multiple times and will lay up to 6 batches of 100-150 eggs during their lifetime (Service 2000). *M. domestica* is diurnal and adult activity consists mostly in seeking food and water, feeding, mating, resting and oviposition (Diether 1976). House flies are multivoltine and go through 10-12 generations annually in temperate regions with populations peaking in summer. The fly does not migrate with the seasons or go into diapause during winter but survives and continues to breed in refuges. Sites utilized for overwintering include barns and other animal-associated locations that are warm enough and offer sufficient development sites and food to support the flies' lifecycle (Black and Krafsur 1986, Kettle 1995).

House flies develop as larvae in a wide range of decaying organic matter such as human and animal feces, rotting vegetation, garbage and carrion. Each of these substrates has a rich microbial community, which supports the proper development of the larvae (Zurek et al. 2000). The larval midgut is especially well suited for digestion of bacteria by a number of attributes. The mid portion of the midgut has a low pH of 3.1, and muscid flies are the only known

invertebrates to have such an acidic midgut (Espinoza-Fuentes and Terra 1987, Terra et al. 1988). The low pH is optimal for the function of lysozyme, which is secreted by the fly for bacterial breakdown (Lemos et al. 1991, Terra and Ferreira 1994). Other enzymes such as pepsin, amylase, maltase and trypsin are secreted, which function to effectively digest bacteria (Terra et al. 1988, Jordao and Terra 1991).

House flies are a significant nuisance pest due to the high populations their and synanthropic nature. Further, they are recognized as efficient mechanical vectors of a number of parasites/pathogens including protozoans, viruses and bacteria (Greenberg 1965, Graczyk et al. 2001). Examples of pathogenic bacteria that have been isolated from *M. domestica* include *Samonella* spp. (Bidawid et al. 1978, Mian et al. 2002), *Shigella* spp. (Bidawid and Edesen 1978, Levine and Levine 1991), *Klebsiella* spp. (Fotedar et al. 1992, Sulaiman et al. 2000), *Escherichia coli* O157:H7 (Grubel et al. 1997, Buma et al. 1999, Kobayashi et al. 1999, Sasaki et al. 2000, Kobayashi et al. 2002, Alam and Zurek 2004), *Vibrio cholerae* (Escheverria et al. 1983, Fotedar 2001), *Campylobacter fetus* (Rosef and Kapperud 1983), *Aeromonas caviae* (Nayduch et al. 2001) and enterococci (Macovei and Zurek 2006, Graham et al. 2009). Several of the above examples include enteric pathogens due to the attraction of the fly to human/animal waste (Greenberg 1965, Bidawid and Edesen 1978, Echeverria et al. 1983, Graczyk et al. 2009, Ahmad et al. 2011).

A number of studies have directly observed the ability of house flies to transmit bacteria/pathogens. Flies infected with *E. coli* O157:H7 were able to contaminate beef and potatoes in laboratory assays (Kobayashi et al. 2002). Another study involving *E. coli* involved quantifying contamination on various foods by *M. domestica* in the lab. Flies successfully contaminated milk, steak and potato salad with *E. coli* at a rate of 43%, 53% and 62% of samples

with counts of 10^3, 10^4 and 10^2 CFU, respectively (De Jesus et al. 2004). Ahmad et al. (2007) exposed eight calves to *E. coli* O157:H7 positive house flies for 48 h. Within 24 h after exposure, fecal samples of all eight calves and drinking-water samples from five of eight calves were positive for the bacterium. A lab-based study revealed that house flies readily contaminate ready-to-eat food with enterococci (Macovei et al. 2008). The authors observed that as few as five flies in as little as 30 minutes (the fewest flies and shortest time tested) resulted in an average of 3.1 x 10^3 enterococci deposited on the food. Also, house flies contaminated milk with *Toxoplasma gondii* oocysts after contacting cat feces that contained the oocysts in laboratory assays (Wallace 1971).

Other studies have provided indirect evidence of house flies as disease vectors. Emerson et al. (1999) conducted a field study that revealed a strong correlation between house flies and the incidences of trachoma and diarrhea among villagers in Gambia. They observed cases of trachoma (an eye disease caused by *Chlamydia trachomatis*) and diarrhea among children (causative organism not identified though suspected to be *Shigella* spp.) before and after fly control efforts with deltamethrin. The study was done for two successive years with two pair of villages, one of the pair with fly management and the control village without fly control. Fly control efforts resulted in a 75% reduction of flies, 75% reduction in new cases of trachoma and 22% fewer diarrheal cases. Levine and Levine (1991) reviewed several studies conducted in the US involving the association of house flies and *Shigella* spp. and noted a correlation of fly activity and dysentery incidence as well as a marked reduction in cases when flies were controlled. Similar reduction of shigellosis was observed among troops in military camps where fly control efforts were carried out (Cohen et al. 1991). Finally, house flies were implicated in an outbreak of *E. coli* O157:H7 in rural western Japan. Flies were collected at a number of

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locations near a dairy farm including a nursery school where a number of humans had become infected (Moriya et al. 1999). A number of potential sources of *E. coli* O157:H7 were tested at the school including food, water, play areas, drainage and resident foul feces and the bacterium was not found. Three separate surveys of house flies resulted in 2.5 – 8% testing positive for *E. coli* O157:H7. Molecular analysis of the isolates from flies and those recovered from patients indicated the same strain of bacterium. Therefore, it was concluded that flies, particularly house flies, were likely responsible for the outbreak.

House flies associated with food animal operations frequently carry microbes that match those in the waste of the respective animals. Ahmad et al. (2011) used multiple genotypic and phenotypic techniques to compare enterococci from house flies and pig feces at two commercial swine operations and observed matching profiles between the two sources. Literak et al. (2009) correlated the antibiotic resistance profiles of *E. coli* from swine feces and those recovered from house flies. Flies from two swine operations in the Czech Republic had matching antibiotic resistance patterns and pulsed-field gel electrophoresis (PFGE) genotype profiles from swine feces. *M. domestica* collected at and near poultry operations had genotypic and phenotypic AR profiles among enterococci that matched those from poultry litter (Graham et al. 2009). Viable *Salmonella enteritidis* were recovered from flies at poultry operations (Mian et al. 2002). And as mentioned above, flies associated with dairies have carried *E. coli* with matching genotypic profiles of those in cattle manure (Buma et al. 1999, Moriya et al. 1999).

There are a number of attributes of the house fly that contribute to its ability to function as a mechanical vector, namely its feeding habits, abundance and close association with humans as well as the nature of larval developmental sites and dispersal habits.

Mouthparts and feeding. House flies are non-biting flies with sponging mouthparts. The proboscis of the fly consists of three primary parts, the rostrum, haustellum and labellum. The labellum is the distal portion of the proboscis and makes direct contact with food/water sources (Dethier 1976). On the ventral surface of the labellum are small grooves known as pseudotracheae and near the food canal are minute prostomal teeth, used to scrape and mechanically break down food sources (Iwasa 1983). While feeding, the fly secretes digestive enzymes that break down solid food into a liquid that can be taken in through the labellum and into the foregut/crop. The labellum is a site of frequent contact with microbes associated with food sources and can serve to disseminate microbes the fly has contacted. Further, studies involving *Escherichia coli* O157:H7 have revealed that the pathogen not only resides in the digestive tract, at least transiently, but may also proliferate in the gut (Kobayashi 1999, Sasaki 2000). This proliferation and enhanced dissemination of pathogens has led to house flies being termed a "bioenhanced vector" to differentiate this from simple mechanical transmission (Kobayashi et al. 1999). The alimentary canal of the flies includes a highly modified crop that branches from the stomadaeum and extends to the abdomen. The crop is a bivalved sac believed to function primarily for storage of sugars utilized for flight (Singh and Judd, 1965). The crop of the house fly has been observed as an important site of bacterial accumulation (Kobayashi et al. 1999, Sasaki et al. 2000, McGaughey and Nayduch 2009). Further, the crop is important in *M. domestica* because of the fly's method of feeding. Because the fly regurgitates when feeding, any bacteria present in the crop are readily deposited on the flies' food source (Graczyk et al., 2001). Flies also frequently defecate on food sources and microbes that have survived to the rectum are passed in this way as well (Kobayashi et al. 1999, Sasaki et al. 2000, Graczyk et al. 2001).

Due to the ability of various microbes/pathogens to proliferate in the house fly digestive tract, a number of studies have focused on tracking the fate of select bacteria in the fly gut in laboratory assays. Kobayashi et al. (1999) fed adult house flies trypticase soy broth containing two strains of *E. coli* O157:H7 at a concentration of 10^9 CFU/ml. Within 1 h of exposure the flies excreted the bacteria and 10^6-10^7 CFU/fly were recovered from the alimentary canal. The flies continued to harbor *E. coli* O157:H7 for up to 72 h. Additionally, they successfully contaminated their substrates with 10^7 CFU/fly at 1 h down to 10^2 at 72 h. The authors observed evidence of the bacteria actively proliferating on the labellum and in the crop (Kobayashi et al. 1999). A study involving the persistence and transmission of *Aeromonas caviae* by house flies was conducted by Nayduch et al. (2002). In this study, the flies were fed 1.2 x 10^4 of the bacterium and were sampled at multiple intervals during the first 24 h and each 24 h thereafter for up to 12 days. The counts of *A. caviae* recovered from the midgut consistently increased to a peak of 8.9 x 10^4 at 48 h, indicating active proliferation in the fly alimentary canal. From day 2-8 the CFU counts were significantly lower and none were recovered after day 8. Groups of six house flies were exposed to ground chicken and actively contaminated the meat from day 1 to 7 of the experiment. McGaughey and Dayduch (2009) conducted a series of lab experiments to observe the fate of *Aeromonas hydrophila* in *M. domestica*. Two strains (motile and nonmotile) of green fluorescent protein-producing *A. hydrophila* were fed to the flies. Viable bacteria were observed in the crop as well as the midgut at 0-22 h. Bacteria counts recovered from the flies at 2 h post infection resulted in a 1000 fold increase of the bacteria, indicating active proliferation in alimentary canal. The bacteria were lysed in the posterior midgut and no viable cells were recovered in excreta. By 24 h no bacteria were observed in the flies, however, the flies were not fed after bacterial infection and the authors postulated that the bacteria would have survived

longer had the flies been actively feeding (McGaughey and Dayduch 2009). In fact, in the previously described assay (Nayduch et al. 2002) involving *A. caviae*, the flies were fed following exposure and the bacteria were recovered up to 8 days. These studies underscore the potential role of house flies as vectors of pathogenic bacteria. It was observed that the bacteria survive transiently in the fly alimentary canal, actively proliferate and are frequently deposited in vomitus and in some cases in excreta as well.

Abundance and association with humans. *M. domestica* is one of the most abundant insect species and is closely associated with humans (synanthropic). They are abundant in environments such as open markets, fairs, restaurants, refuse dumps, animal pens, confined animal feeding operations and in homes (Echeverria et al. 1983).   Due to their abundance, association with humans and attraction to both filth and human food their role as disease vectors is enhanced.

Larval substrates and microbes. House fly larval development substrates include a variety of rotting organic matter, which is rich in microbial communities. The fly larvae are constantly contacting and consuming the associated microbes/pathogens and are able to carry pathogens from larval substrates through pupation to adult eclosion (Greenburg 1965, Rochon et al. 2005). Further, adult house flies aggregate at sites of larval development as well for breeding, oviposition and feeding and can easily acquire associated pathogens (Blackith and Blackith 1993).

Dispersal. House flies are known to disperse great distances often with no apparent patterns with regard to wind direction, food/water proximity or suitable mating and larval development sites. A mark and recapture study of wild house flies in rural Georgia resulted in flies captured up to 8 km from the release point in 24 h (Quarterman et al. 1954a). The same

authors conducted a similar study in an urban area of Georgia (Quarterman et al. 1954b). In this setting flies were captured up to 12 km from release points. During both of these studies it was noted that fly dispersal was random from various release points and occurred even in the presence of adequate food and larval breeding habitats. Murvosh and Thaggard (1966) conducted studies of house flies dispersion and also noted random dispersal patterns. A survey of *M. domestica* captured around a 2.5 million chicken egg laying facility in rural Ohio pointed to the farm as the primary source of flies and they regularly dispersed from the facility up to 6.4 km (Winpisinger et al. 2005). A study done in and around Manhattan, KS estimated house flies dispersion by use of multilocus DNA fingerprinting and by the profile of antibiotic resistance among the enterococci in the gut of the flies (Chakrabarti et al. 2010). The authors noted a high migration rate based on these two parameters and attributed livestock production facilities as a primary source for flies based on the antibiotic resistance profiles of the bacteria. They concluded that flies could disperse up to 125 km from these operations and could potentially spread the resistant microbes they harbored.

In addition to the potential for microbes to proliferate in the digestive tract of house flies, studies have revealed that horizontal transfer of mobile genetic elements and genes for toxins and antibiotic resistance can occur among bacteria in this environment. Petridis et al. (2006) observed relatively frequent transfer of genes for chloramphenicol resistance and Shiga toxin among donor and recipient strains of *E. coli* in both the midgut (transconjugant/donor 10^{-2}) and crop (10^{-3}) of *M. domestica* after 1 h. In another study, a gene for tetracycline resistance associated with a pheromone-responsive plasmid was transferred between *Enterococcus faecalis* strains in the house fly digestive tract (Akhtar et al. 2009). Transconjugants were observed beginning at 24 h and continued throughout the study up to 5 days at a transconjugant/donor rate

of 8.6 x 10^{-5} up to 4.5 x 10^{1}. The implications of these studies are significant to public and veterinary health as they point to the ability of bacteria to actively disseminate toxins and antibiotic resistance genes within the *M. domestica* gut beyond what is consumed initially by the fly.

1.2 BACTERIAL ANTIBIOTIC RESISTANCE

The burden posed by antibiotic resistant (AR) bacteria is increasing worldwide (Levy and Marshall 2004, Hawkey 2008). The rise of AR pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) has increased morbidity and mortality associated with bacterial infection and made effective treatment a significant challenge (Rice 2006, Amyes 2007, Reik et al. 2008). Costs associated with AR bacterial infections include increased patient care and treatment expenses and the need for newer, often higher-priced antimicrobials to treat the most resistant infections (Hawkey 2008). Unfortunately, at the same time the number of new antibiotics being approved and introduced into the market has steadily declined during recent decades (down 54% from 1983 to 2002) (Spellberg et al. 2004, Tenover 2006, Taubes 2008). Clearly, better management of antibiotic resistance is needed, as is enhanced knowledge of the ecology of AR strains and associated resistance genes.

Exposure of bacteria to antimicrobials can select for those microbes that are intrinsically resistant, have acquired AR genes, or have a mutation that makes them resistant. The pressure applied to the microbial population by the antimicrobial eliminates the susceptible strains, leaving behind the resistant cells and over time the resistant microbes predominate (Levy 2002, Hawkey 2008). Two such environments where antibiotic selection pressure is intense are the clinical, such as hospital intensive care units (ICU) and agricultural settings related to food

animal production. Hospital ICUs are an ideal environment for the emergence, proliferation and

maintenance of AR bacteria. Patients receive extended treatment with the majority being

administered multiple broad spectrum antibiotics (Iredell and Lipman 2005). Certain bacteria

have adapted to this environment and established nosocomial strains that pose a significant

challenge both for treatment and containment (Hawkey 2008). For instance, clinical strains of

enterococci have emerged that are multiple drug resistant as well as tolerant of common

disinfectants such as alcohol, chlorine and glutaraldehyde, which allow them to better survive on

various surfaces such as equipment, counters, bed rails, door handles, etc. (Top et al. 2007).   The

hardiness of enterococci and ability to survive in the low-nutrient environments facilitates their

persistence and vertical transfer among patients (Bonilla et al. 1996, Lleo et al. 1998, Heim et al.

2002). A survey done in 2000 of 1391 high-risk patients at a Chicago teaching hospital revealed

that 188 (13.5%) had antibiotic-resistant infections (ARI) and of these 188, 135 (72%) had

hospital-acquired infections (Roberts et al. 2009). The authors estimated that the ARI resulted in

a 6-13 day longer stay in the hospital at a cost of $19,000-29,000 per patient and resulted in 11

deaths.   Another prime environment for the emergence of AR bacteria are food animal

production systems. Silbergeld et al. (2008) listed four reasons why the use of antimicrobials in

food animal production is a significant contributor to antimicrobial resistance, 1) a large quantity

of antibiotics are administered, the majority for growth promotion, 2) abundant subtherapeutic

exposure of microbes to the agents both in the animals and passed with manure into the

environment, 3) all major classes of antibiotics are used and 4) human exposure by those

handling the livestock and through consumption of food products. In 2010, the FDA provided

the first estimate of antibiotics sold for use in food animal production. In that report, an

estimated 15 million Kg of active ingredients were sold in 2009. Among the classes of

antibiotics, tetracyclines were the most abundant at 5.1 million Kg (USFDA 2010). There is evidence that the AR bacteria and genes that are promoted in animals exposed to antibiotics are transferred to other environments. A study focused on antibiotic resistant bacteria from archived soil samples collected from 1940 to 2008 in the Netherlands revealed a significant increase, primarily for resistance genes to β-lactamases and tetracyclines over the period (Knapp et al. 2010). The authors attributed the rise in AR gene prevalence primarily to agricultural use of antibiotics for growth promotion from waste discarded and contaminating the soil. Further, AR genes have been detected in diverse water sources such as rivers, ponds, dairy lagoons and ditch water (Pruden et al. 2006, Koike et al. 2007).

The modes of actions by which antibiotics affect bacteria are limited primarily to four targets: 1) cell wall synthesis (beta-lactams, glycopeptides), 2) inhibition of protein synthesis (aminoglycosides, tetracyclines, macrolides, streptogramins) 3) interference with nucleic acid synthesis (fluroquinolones, nitrofurans) and 4) inhibition of metabolic pathways (sulfonamides) (Tenover 2006). All modes capitalize on differences between prokaryotes and eukaryotes; the peptidoglycan cell wall of prokaryotes, rRNA of prokaryotes (60S versus 80S in eukaryotes) and differences in topoismerases in the case of nucleic acid synthesis inhibition. Targeting these specific sites in prokaryotes is important for limiting potential damage to the host (human, animal). The lack of diverse antimicrobial targets can also enhance the speed with which resistance can emerge and spread for new agents as there is considerable cross resistance to antimicrobials in the same class by several resistance determinants. Moreover, because many of these agents are heavily used in both agriculture and clinical settings there is further potential proliferation of resistance genes via horizontal transfer (Martins da Costa et al. 2006, Aminov et al. 2007, Schluter et al. 2007, Aminov et al. 2007, Knapp et al. 2010).

Resistance to antibiotics among bacteria can be broadly categorized as intrinsic or acquired. Intrinsic resistance involves an innate ability of the bacterium to overcome or avoid the action of the antibiotic (Huycke 1998, Schluter et al. 2007, Top et al. 2008). Intrinsic resistance usually involves chromosomal genes and is typically not transferrable to other bacteria (Huycke 1998, Franz et al. 2003). Acquired resistance, as the name suggests, involves a bacterium that was previously susceptible to an antibiotic agent that gains resistance. The two methods of acquired resistance are by mutation of existing genes or horizontal acquisition of resistance genes from other bacteria (Dzidic and Bedekovic 2003). Horizontal transfer of resistance determinants is attributed to approximately 95% of resistance (Nwosu 2001) and occurs primarily through three means; transformation, transduction or conjugation (Davison 1999, Rice 2000, Nwosu 2001, Tenover 2006). Transformation involves the uptake of naked DNA released from a lysed bacterium. Transduction occurs when a bacteriophage, a virus that infects bacteria, encapsulates DNA from a host bacterium rather than its own and transfers to another bacterium. If the DNA includes AR determinants the gene(s) can be relayed to another bacterium. Conjugation involves cell to cell transfer of genetic material.

There are three primary methods of acquired antibiotic resistance, 1) enzymes that modify the antibiotic, 2) modification of the antibiotic target and, 3) the use of efflux pumps that expel the agent (Tenover 2006). A number of resistance genes code for enzymes that degrade the antimicrobial agent before it can cause damage to the cell. A common example is the genes that code for acetyltransferases (*aac*), which provide resistance to aminoglycosides (e.g. gentamicin) by enzymatically modifying the agent (Klare et al. 2003). These genes are common among aminoglycoside-resistant gram positive bacteria. Additionally, the target of the antimicrobial can be modified to reduce the effectiveness of the agent. The gene cluster *vanA*,

which results in high level resistance to glycopeptides (vancomycin) is an example. Glycopeptides target the peptide cross-linking of peptidoglycan in the cell wall by binding to the D-Ala-D-Ala terminal amino acid sequence. The *vanA* operon induces modification of the terminal sequence to D-Ala-D-Lac, which significantly reduces the binding affinity of glycopeptides (Kak and Chow 2002). Finally, examples of multi-drug efflux pumps include NorA among *S. aureus* and EmeA among enterococci (Jonas et al. 2001). The pumps actively expel various drugs from the respective cell before they can act on the DNA of the bacteria (van den Bogaard 1997).

Mobile genetic elements (MGE), including plasmids, transposons and gene cassettes are responsible for the mobilization of many AR genes (Dzidic and Bedekovic 2003, Schluter et al. 2007, Hegstad et al. 2010). These elements are highly mobile and able to relatively quickly confer determinants such as antibiotic resistance both intra and interspecifically. Plasmids are circular, transferrable extrachromosomal genetic elements and are common among bacteria and range in size from 1 to over 1,000 kbp (Weaver et al. 2002. Sorensen 2005, Hegstad et al. 2010). They contain a variety of accessory genetic material including AR genes as well as functional genes used in plasmid replication and transfer. Plasmids are classified by such criteria as replication mode (rolling circle plasmids), compatibility (Inc18 plasmids) or method of transfer (pheromone responsive plasmids) and can be narrow or broad in host specificity (Weaver et al. 2002, Sorensen 2005, Hegstad et al. 2010). Transposons are known as jumping genes, which have specific coding sequences that produce transposases involved in movement from and to the same or different genomes. Transposons may move directly from one cell to another (conjugative transposons) or do so via an intermediary such as a plasmid (Tn3-family transposons) (Sood et al. 2008). Several transposons harbor antibiotic resistance genes and

therefore play a substantial role in their ecology (Weaver et al. 2002, Hegstad et al. 2010). Due to linking of resistance genes to the same MGE, use of one antibiotic can lead to multiple drug resistance and mobilization of resistance genes in the absence of direct pressure (Levy 2002). Additionally, many antibiotics pass through human or animal digestive tracts without breakdown and therefore can act as environmental contaminants that induce expression of AR genes and horizontal transfer of AR genes (Levy 2002). Though bacteria are adapted to specific environments, many are readily dispersed through media such as soil and water where there is significant interaction with other bacteria, often accompanied by genetic exchange including AR genes (Nwosu 2001).

1.3 THE ENTEROCOCCI

Enterococci are gram positive bacteria in the phylum Firmicutes, class Bacilli, order Lactobacillales, family Enterococcaceae and genus *Enterococcus* (Schleifer and Kilpper-Balz 1984). They are facultative anaerobic, lactic acid cocci and are tolerant of a wide range of temperatures, pH and nutrient-poor environments (Lleo et al. 1998, Heim et al 2002, Fisher and Phillips 2009). The primary niche of enterococci is as commensals of the digestive tract of a wide range of animals including humans. *E. faecalis* and *E. faecium* are two of the most prevalent species and are of most medical and veterinary significance. Other common species include *E. gallinarum*, *E. casseliflavus*, *E. mundtii*, *E. malodoratus*, *E. hirae* and *E. flavescens*. *E. mundtii* and *E. casseliflavus* are two species commonly associated with plants (Klein 2003). Among the human gut microbiota, *E. faecalis* and *E. faecium* are the most common enterococcal species and account for approximately 1% of the total bacteria with a concentration of 10^2-10^8 CFU/g of feces (Ogier and Serror 2008, Fisher and Phillips 2009). Among food production animals, *E. faecium*, *E. faecalis* and *E. hirae* are common with *E. gallinarum* occasionally

present (Klein 2003). Enterococci are widespread among insects as well. Martin and Mundt (1972) sampled 403 insects from the orders Coleoptera, Diptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera, and Orthoptera for enterococci. Overall, 53% were positive for the bacteria; *E. faecalis*, *E. faecium* and *E. casseliflavus* were the most abundant species recovered with a mean concentration of 2.0×10^2 to 3.9×10^7 CFU per insect.

Over the past few decades enterococci have emerged as the third most common nosocomial pathogens overall after *Escherichia coli* and *Staphylococcus aureus* (French 1998, Arias and Murray 2009). Specifically, they are the second most common bacteremia pathogen and third most common for urinary tract and surgical site infections (Hidron 2008). As previously mentioned, *E. faecalis* and *E. faecium* are responsible for the majority of infection with an estimated rate of 60/40% among the two species, respectively (Top 2007, Hidron 2008). Factors contributing to the significance of enterococci as nosocomial pathogens include their hardiness, intrinsic AR, ease for horizontal acquisition of resistance determinants and the presence of various virulence factors.

Antibiotic resistance among enterococci has been observed to every major class of antibiotics, either intrinsically or acquired. Enterococci are intrinsically resistant to low concentrations of *β*-lactams, quinolones, lincosamides and aminoglycosides (Landman and Quale 1997, Ogier and Serror 2008, Top et al. 2008) and *E. casseliflavus*, *E. flavescens* and *E. gallinarum* are intrinsically resistant to low level glycopeptides (Domig et al. 2003). Resistance is commonly acquired to *β*-lactams (methicillin, ampicillin), macrolides (erythromycin), lincosamides, tetracyclines (tetracycline, doxycycline), aminoglycosides (streptomycin, gentamicin) and streptogramins (quinupristin/dalfopristin) and increasingly to glycopeptides (vancomycin) (Kak and Chow 2002, Arias and Murray 2008).

There are a number of plasmids and transposons that have been identified that are responsible for conferring resistance genes. Three types of plasmids are common among enterococci, the Inc18 plasmids, rolling circle replicating plasmids and pheromone responsive plasmids (Weaver et al. 2002). The Inc-18 plasmids have a wide host range, were the pheromone responsive plasmids (PRP) are transferred intraspecifically. There are approximately 20 PRP among *E. faecalis* (Wirth 1994) and one recently identified for *E. faecium* (Hegstad et al. 2010). Plasmid transfer is induced by release of a peptide signal from the plasmid-free recipient to induce the expression of binding proteins on the donor. The donor is then able to selectively bind to recipient cells to carry out conjugative transfer of the targeted plasmid (Wirth 1994). Three types of transposons are present among enterococci, Tn*3* transposons, composite transposons and conjugative transposons. Some well-studied transposons among these groups include (transposon group/resistance) Tn*917* (Tn*3*/macrolide), Tn*916* (conjugative/tetracycline), Tn*1545* (conjugative/tetracycline and macrolide) Tn*5385* (composite/aminoglycoside) and Tn*1546* (Tn*3*/glycopeptide) (Weaver et al. 2002, Top et al. 2008, Hegstad et al. 2010).

A number of virulence factors are associated with clinically significant enterococcal strains that aid in avoidance of host immune responses and/or breakdown of host tissue. The best studied of these are gelatinase (Gel), cytolysin (Cyl), aggregation substance (AS) and enterococcal surface protein (Esp) (Gillmore et al. 2002, Hancock and Gillmore 2006, Ogier and Serror 2008, Upadhyaya et al. 2009). Gelatinase is a metalloprotease that causes degradation of host tissue and is associated with formation of biofilm (Hancock and Gillmore 2006, Mohamed and Huang 2007). Cytolysin is a bacteriocin that also displays hemolytic activity and is associated with enterococci causing bacteremia. Aggregation substance is a surface-localized protein expressed among *E. faecalis* and is involved in pheromone responsive plasmid

conjugation. Aggregation substance is also suspected to play a role in binding to eukaryotic cells and is positively associated with urinary tract infections (UTI). Additionally, AS plays a putative role in binding and traversing the gut barrier (Waters et al. 2003). Enterococcal surface protein is another surface protein which plays a role in biofilm formation and UTI (Gillmore et al. 2002, Hancock and Gillmore 2006, Mohamed and Huang 2007). A synergistic effect of virulence involving endocarditis has been shown among enterococci that contain both AS and Cyl (Chow et al. 1993). Both of these virulence factors are frequently transferred horizontally among *E. faecalis* on pheromone responsive plasmids (Hegstad et al. 2010). Additionally, Esp, AS and Cyl are frequently found on pathogenicity islands, a large chromosomal region that encodes virulence (Ogier and Serror 2008, Upadhyaya et al. 2009).

The advent of enhanced genetic and diagnostic techniques such as Multi Locus Sequence Typing (MLST) allows comparison of bacterial genotyping results obtained among different laboratories (Urwin and Maiden 2003). As a result, epidemiological databases of enterococci have been developed as well as identification of pathogen clonal groups, which has been particularly helpful in assessing the spread and extent of various hospital-adapted strains. These strains are often multiple-antibiotic resistant and express several virulence factors, making them distinct from commensal strains (Huycke et al. 1998, Top et al. 2008). It is speculated that nosocomial strains emerged due to the hardiness of enterococci and ability to acquire antibiotic resistance and virulence factors under the selection pressure of extensive antibiotic use (Huycke et al. 1998, Top et al. 2008).

Of particular concern is the rise of vancomycin resistant enterococci (VRE), the majority of which are *E. faecium*. VRE were estimated to be responsible for close to 21,000 human infections annually in US hospitals during 2003 and 2004 (Reik et al. 2008). Vancomycin

resistance is of particular concern because this antibiotic was until recently the drug of last resort for treating AR Gram positive bacterial infections (Huycke et al. 1998). The first identified case of VRE among US patients was in 1988; infections increased through the 1990's and at present VRE account for 80% of clinical *E. faecium* infections (Willems and van Schaik 2009). An *E. faecium* genogroup, clonal complex 17 (CC17), has been identified. It is estimated that CC17 has been circulating among hospitals since the early 1980's (Galloway-Pena et al. 2009). This subpopulation is known for high level ampicillin and vancomycin resistance and presence of several putative virulence factors including Esp (Top et al. 2008, Willems et al. 2009).

Antibiotic resistant and virulent enterococci are also common among food production animals due to abundant use of antibiotics used for growth promotion (Thal et al. 1995, Silbergeld et al. 2008). Further, evidence exists that resistant enterococci and other microbes associated with animal waste enter other environments such as groundwater, lakes and rivers (Krapac et al. 2002, Koike et al. 2007, Silbergeld 2008).

In addition to being opportunistic pathogens, enterococci are also considered reservoirs of antibiotic resistance genes, thus playing an important role in AR gene ecology. Horizontal gene transfer (HGT) has been identified as a way that genes are conferred both intra and interspecifically and can bridge ecological barriers that the bacteria themselves may not be capable of (Kotzamanidis et al. 2009). Horizontal gene transfer involving enterococci has been observed in the digestive tract of humans (Lester et al. 2006), mice (Dahl et al. 2007, Moubareck et al. 2003), rats (Jacobsen et al. 2007) and house flies (Akhtar et al. 2009) and in wastewater during treatment (Marcinek et al. 1998). A primary concern of HGT is that AR genes can spread to other enterococci or more pathogenic bacteria such as *Staphylococcus aureus* (Huycke et al. 1998, Sung et al. 2007, Fisher and Phillips 2009).

1.4 ASSOCIATION OF HOUSE FLIES AND ENTEROCOCCI

A few studies have specifically considered the association of *M. domestica* and enterococci. Graham et al. (2009) were interested in the potential role of house flies to acquire and transfer AR enterococci from broiler poultry operations. They isolated and characterized enterococci from poultry litter at three operations and from flies both onsite and up to 3.2 km away in a tristate region (Delaware, Maryland and Virginia). The genotypic and phenotypic AR profiles of enterococci from litter and house flies matched well, suggesting that the flies play a role in the ecology and dissemination of enterococci and associated resistance genes at these operations (Graham et al. 2009). Enterococci were also characterized from house flies, German cockroaches and swine feces at two swine operations in Kansas and North Carolina (Ahmad et al. 2011). Ninety four percent of house flies were positive for enterococci with a mean concentration of 10^4 CFU/fly. *E. faecalis* was the most abundant species recovered from flies and *E. hirae* was most common from swine feces. Both sources (flies and swine feces) had similar phenotypic and genotypic AR profiles as well as AR genes and MGE. Genotyping of *E. faecalis* and *E. faecium* isolates using PFGE revealed clonal matches among the bacteria from feces, house flies and roaches. Another study assessed the AR profiles of enterococci from house flies captured in rural and urban environments as part of an effort to assess fly migration up to 124 km to and from Manhattan, KS (Chakrabarti et al. 2010). The authors noted considerable migration of flies based on multilocus DNA fingerprinting. Over 90% of the flies were positive for enterococci and the AR prevalence among the bacteria was highest among flies captured at cattle feedlots followed by dairies and urban sites such as restaurants. There was a positive association of AR prevalence of enterococci from house flies to distance of restaurants at which the flies were captured from feedlots. Thus, the authors attributed the feedlots as the

primary source of AR enterococci. Macovei and Zurek (2006) sampled the digestive tracts of house flies for enterococci at five fast-food restaurants in Northeastern Kansas and found AR enterococci common among the *M. domestica*. Ninety seven percent of the flies were positive for enterococci with a mean CFU of 3.1 x 10^3/fly. Two hundred and five of the enterococcal isolates cultured were characterized. *E. faecalis* was the most abundant species (88.2%) and AR phenotypic tests revealed resistance to tetracycline (66.3% of isolates), erythromycin (23.8%), streptomycin (11.6%) ciprofloxacin (9.9%) and kanamycin (8.3%). Further, several virulence genes were identified among the isolates including *gelE* (70.7%) *asa1* (33.2%), *esp* (8.8%) and *cylA* (8.8%) (Macovei and Zurek 2006). A follow-up study found food from the same restaurants was commonly contaminated with AR enterococci (Macovei and Zurek 2007). Three ready-to-eat food items (chicken salad, chicken burger, and carrot cake) were sampled in summer and winter. Overall concentration of enterococci throughout the year averaged 10^3 CFU/g with greater prevalence during the summer (92.0% of salads, 64.0% of burgers) than the winter (64.0% of salads, 24.0% of burger sample). Enterococci from summer samples were resistant to tetracycline (22.8%), erythromycin (22.1%) and kanamycin (13%). The higher prevalence of enterococcal contamination among food samples in the summer correlates with higher *M. domestica* activity, thus indirectly implicating the fly as at least a partial source of contamination. This study implied that food served in restaurants is commonly contaminated with AR enterococci and that flies may play a role in this contamination (Macovei and Zurek 2007). Another study directly assessed the ability of *M. domestica* to contaminate ready-to-eat food with enterococci under laboratory conditions (Macovei et al. 2008). The authors observed that as few as five flies in as little as 30 minutes (the fewest flies and shortest time tested) would result in an average of 3.1 x 10^3 enterococci deposited on the food.

These studies demonstrated either direct or indirect evidence that an agricultural source (poultry, swine and cattle feedlot operations) were the source of the AR enterococci and that house flies were a likely vector. Indeed, the interface of microbes/pathogens of food production animal origin and house flies has been well established (Rosef and Kapperud 1983, Buma et al. 1999, Iwasa et al. 1999, Mian et al. 2002, Graham et al. 2009, Chakrabarti et al. 2010) and due to the abundant access *M. domestica* has to numerous sources of animal waste, this is likely the primary source of enterococci, particularly in rural areas.

Another potential source of AR enterococci in house flies involves a human/clinical source, specifically that of clinical origin where there is considerable antibiotic pressure. Though it is unlikely that flies gain significant access to hospitals or health clinics directly or indirectly via medical waste in developed countries, sewage waste from these sources is a potential point source at municipal wastewater treatment facilities. This may be a significant source of microbes for house flies, particularly in urban settings. In particular reference to AR enterococci, a human source may represent a greater public health threat than animal sources. If these bacteria were ingested by humans they would likely easily multiply with no ecological barriers unlike enterococci of animal origin. Further, once established in the human gut, they would likely convey AR determinants to commensal microbiota, establishing a reservoir of AR genes in the human GI tract. The significance of WWTF as a source of AR enterococci for flies has not been addressed. Focusing on this environment will lead to a better understanding of the potential human/clinical sources of these bacteria and the role of flies in their ecology.

1.5 WASTEWATER TREATMENT AND ASSOCIATED MICROBIAL ECOLOGY

A 2007 report from the North East Biosolids and Residuals Association (NEBRA) estimated that there were approximately 17,000 operating wastewater treatment facilities (WWTF) in the US (NEBRA 2007). Wastewater treatment operations vary considerably regarding specific design; however, the following is a summary of the key processes in activated sludge treatment, the most common technology utilized for wastewater treatment (Cheremisinoff 1996, Horan 1990). Raw sewage (influent) is usually passed through initial bar screening to remove large insoluble items such as paper, large food particles and other debris (Fig 1.1). Often the next stage of initial treatment is the removal of small particle insoluble grit such as sand and fine gravel before passing to a primary clarifier. In the clarifier the waste flow is significantly reduced; the top layer is skimmed to remove oils and other floating material and solids are allowed to settle. From the clarifier, the liquid and solid wastes (sludge) are diverted to separate processing flows. Liquid waste is directed to an aerator tank where oxygen is actively pumped into the solution to facilitate aerobic digestion of soluble waste by microbes. Retention time in the aeration tank averages 8-10 h. Often there is a secondary clarifier following aeration for liquids where the remaining solids are allowed to settle. The sludge that settles in the secondary clarifier, known as activated sludge, is recirculated into the influent side of the aeration tank due to the rich community of microbes, which effectively break down suspended and dissolved waste in the aeration tank. Following secondary clarification, liquids are directed to disinfection, commonly utilizing UV or chlorine to eliminate pathogens and reduce coliforms to acceptable levels. Disinfected effluent is generally released to natural bodies of water such as lakes, rivers or coastal waters. The total time to process liquids from influent to effluent takes approximately

12 h. Solids separated during primary clarification are directed to an aerobic or anaerobic sludge

digester for break down (Cheremisinoff 1996, Horan 1990). Digestion is temperature dependent

and ranges from approximately 20 days in the summer to 120 days in winter. Following

digestion, the solid waste (biosolids) is stored before final removal (Fig 1.1). An estimated 5

million metric tons of dry biosolids are produced annually in the US (National Research Council

2002). Forty to Sixty percent of US biosolids are used to fertilize agricultural land; other forms

of disposal include incineration and landfill application (Krauss and Page 1997, National

Research Council 2002).

Wastewater treatment facilities serve as the principal consolidation point for human

waste in industrialized societies and play a significant role in the ecology of numerous microbes.

Many of the bacteria shed in human waste, including those harboring antibiotic resistance, can

end up at the treatment facility. Resistant bacteria have been detected in wastewater raising the

concern that the bacteria and associated AR determinants could be passed to other microbes and

potentially released to the environment through plant effluent and biosolids (Sturtevant and

Feary 1969, Grabow and Prozesky 1973). While many enteric microbes such as strict anaerobes

do not survive well outside of the human digestive tract, many others, including potential

pathogens, have been cultured from wastewater and at various points along the waste treatment

stream including *Escherichia coli* (Sturtevant and Feary 1969, Grabow and Prozesky 1973,

Walter and Vennes 1985) *Salmonella typhi* (Grabow and Prozesky 1973, Schluter et al. 2007),

enterococci (Harwood et al. 2001, Blanch et al. 2003, Poole et al. 2005, Martins da Costa et al.

2006, Ahmed et al. 2008, Beier et al. 2008, Nagulapally et al. 2009, Kotzamanidis et al. 2009),

Acintenobactor spp. (Guardabassi et al. 1998), *Staphylocuccus aureus*, *Legionella pneumophila*,

and *Clostridium difficile* (Viau and Peccia 2009). Waste from environments under substantial

antibiotic pressure such as from hospitals typically contains a greater percentage of single and multiple drug resistant bacteria (Grabow and Prozesky 1973, Guardabassi et al. 1998, Harwood et al. 2001, Blanch et al. 2003, Reinthaler et al. 2003, Kotzamanidis et al. 2009).

In addition to concerns associated with receiving and proliferation of pathogens and antibmicrobial resistant microbes, several studies have reported that horizontal transfer of AR genes occurs. AR microbes from WWTF and/or wastewater have successfully conferred resistance to previously susceptible microbes under laboratory conditions (Grabow and Prozesky 1973, Mach and Grimes 1982, Marcinek et al. 1998, Schluter et al. 2007). Additionally, two of these studies involved detecting horizontal transfer of resistance determinants directly in WWTF environments (Mach and Grimes 1982, Marcinek et al. 1998). In fact, WWTF have been described as a drug resistance gene pool where there is frequent and relatively uninhibited gene exchange among various bacteria (Martins da Costa et al. 2006, Schluter et al. 2007).

The concentration of enteric bacteria is generally reduced during treatment of both liquid and solid waste. Martins da Costa et al. (2006) observed a 0.5-4 log reduction in enterococci in WWTF effluent compared to that of influent. Additionally, Nagulapally et al. (2009), who focused exclusively on AR coliforms, *E. coli* and enterococci, reported a 2-3 log reduction of these following secondary treatment and reduction to non-detectable levels among plant effluent following UV disinfection. Wen et al. (2009) noted 2-3 log reduction of coliforms, enterococci and *E. coli* in a laboratory setting that replicated the secondary treatment process of activated sludge treatment. Farrah and Bitton (1983) observed a 1-2 log reduction of total coliforms, streptococci and *Salmonella* spp. following aerobic digestion of sludge in a laboratory setting.

Other studies have reported a selective increase in AR bacteria during waste treatment leading to the conclusion that wastewater treatment can selectively increase certain resistant

bacteria. Zhang et al. (2009) observed significant increase of *Acinetobacter* spp. resistant to amoxicillin/clavulanic acid (37.9% increase), chloramphenicol (25.2%), rifampin (63.1%) and multi-drug resistant (33.0%). Additionally, although Ferreira da Silva et al. (2006) noted an overall decrease among enterococci during treatment, they reported a significant increase in ciprofloxacin resistant *E. faecium*.

While waste treatment reduces enteric bacteria in most cases, it often does not eliminate them, therefore, plant effluent and digested biosolids can be released into the environment that contain AR bacteria. Ferreira da Silva et al. (2006) sampled treated wastewater from a WWTF in Portugal and found enterococci resistant to ciprofloxacin (33% prevalence), erythromycin (40%) and tetracycline (57%). Further, Iversen et al. (2004) used a genetic approach to find clonal *E. faecalis* in both hospital sewage and surface waters following wastewater treatment in Sweden. Samples of wastewater effluent and downstream in rivers from effluent release contained AR *Acinetobacter* spp. that matched the profile of those found at the WWTF (Zhang et al. 2009). In addition to wastewater effluent, biosolids have also been found to contain and release AR enterococci and other resistant bacteria in environments such as agricultural land and nearby bodies of water (Reinthaler et al. 2003, Selvaratnam and Kinberger 2004, Martins da Costa 2006).

1.6 STUDY GOAL AND OBJECTIVES

Wastewater treatment operations primarily receive human waste including hospital sewage where high prevalence of AR bacteria is common. Antibiotic resistant enterococci are frequently present in sewage and though are reduced during waste treatment, they are not completely eliminated (Iversen et al. 2004, Ferreira da Silva et al. 2006, Martins da Costa et al. 2006, Nagulapally et al. 2009). House flies and other filth flies often have direct and unhindered

access to many steps in the waste processing flow, including bar screening, aeration tanks, clarifiers, sludge digesters and biosolid storage. House flies therefore can acquire bacteria associated with waste both externally on the body and internally in the digestive tract. If the flies then disperse from the WWTF they can carry and possibly disseminate the microbes to other areas, with a potential impact on human health. This route of bacteria dispersal from WWTF is the thrust of the research in this dissertation.

Overall Research Goal: Assess the role of house flies in the dissemination of antibiotic resistant enterococci from wastewater treatment facilities to the surrounding residential environment

There are three specific objectives and hypotheses:

Objective 1: Isolate and characterize enterococci from four wastewater treatment facilities (house flies and sludge/biosolids) and house flies in the surrounding residential environment.

Hypothesis: House flies acquire and disseminate antibiotic resistant enterococci from wastewater treatment facility to the surrounding environment.

Objective 2: Determine the spatial and temporal fate of *E. faecalis* OG1RF in the house fly digestive tract.

Hypothesis: *Enterococcus faecalis* remains viable and multiplies in the house fly digestive tract.

Objective 3: Assess biosludge at a wastewater treatment facility as a larval developmental

habitat of muscoid flies.

Hypothesis: Biosludge at wastewater treatment facilities can serve as a developmental habitat

for muscoid flies.

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1.8 FIGURES

Figure 1.1 Schematic of the activated sludge process of wastewater treatment.
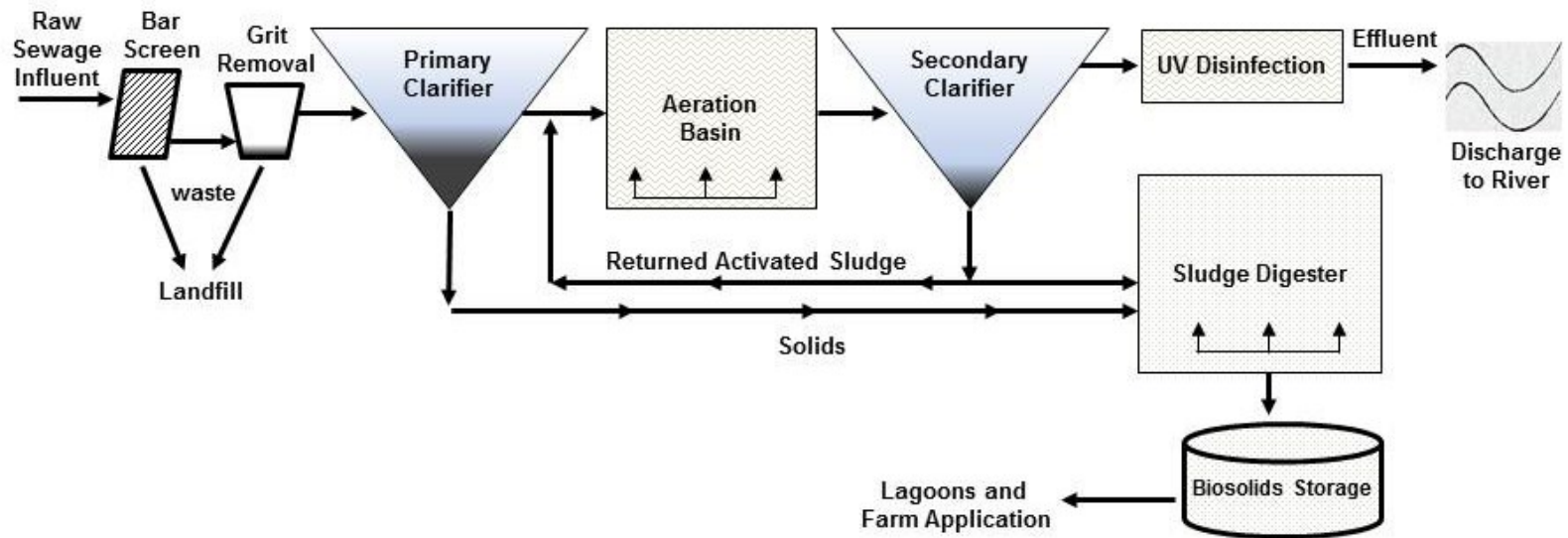
CHAPTER 2: CHARACTERIZATION OF ENTEROCOCCI AT WASTEWATER TREATMENT FACILITIES AND FROM ASSOCIATED HOUSE FLIES

2.1 ABSTRACT

Enterococci are a group of commensal gram positive bacteria that are important

nosocomial pathogens and are commonly antibiotic resistant. The bacteria are abundant in food-

animal production environments, as well as in human sewage and at wastewater treatment

facilities (WWTF). This study involved use of numerous independent measures to assess the

hypothesis that house flies, *Musca domestica* (L.), acquire and potentially disseminate antibiotic

resistant and virulent enterococci from WWTF. House flies and sludge from four WWTF in

northeastern Kansas were sampled for culturing enterococci. The enterococci were quantified,

identified, and screened for antibiotic resistance and virulence traits by phenotypic and genotypic

analysis**.** Of twelve antibiotics screened, enterococci were most commonly resistant to

tetracycline, doxycycline, streptomycin, gentamicin and erythromycin. No enterococcal isolates

were resistant to vancomycin, tigecycline and linezolid. The profiles of enterococci (diversity,

antibiotic resistance and virulence) from WWTF sludge and the house fly digestive tract were

similar, indicating that the flies successfully acquired the bacteria from the substrate.

Enterococci from house flies captured away from Site 1 WWTF were similar in their resistance

profile but the resistance was significantly less prevalent than among enterococci from sludge

and onsite house flies. The greatest amount of enterococci with antibiotic resistance and

virulence factors (gelatinase, cytolysin, enterococcus surface protein, and aggregation substance)

originated from the WWTF that processed meat waste from a nearby commercial sausage plant

(Site 1), suggesting an agricultural rather than human/clinical source of the isolates. Multiple-

resistant *E. faecalis* successfully transferred resistant determinants to recipient *E. faecalis* in both

broth and filter mating assays.   Genotyping with pulsed-field gel electrophoresis of select

enterococci revealed clonal matches among *E. faecalis* from sludge and onsite house flies.

2.2 INTRODUCTION

The burden posed by antibiotic resistant (AR) bacteria is increasing worldwide (Levy and Marshall 2004, Hawkey 2008). The rise of AR pathogens such as methicillin-resistant *Saphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) has increased morbidity and mortality associated with bacterial infection and made effective treatment a significant challenge (Rice 2006, Amyes 2007, Reik et al. 2008). Costs associated with AR include increased patient care and treatment expenses and the need for newer, often higher priced antimicrobials to treat the most resistant infections (Hawkey 2008). Unfortunately, at the same time the number of new antibiotics being approved and introduced into the market has steadily declined during recent decades (down 54% from 1983 to 2002) (Spellberg et al. 2004, Tenover 2006, Taubes 2008). Clearly, better management of antibiotic resistance is needed, as is enhanced knowledge of the ecology of AR strains and associated resistance genes.

Exposure of bacteria to antimicrobials can select for those microbes that are intrinsically resistant, have acquired AR genes, or have a mutation that makes them resistant. The pressure applied to the microbial population by the antimicrobial eliminates the susceptible strains, leaving behind the resistant cells and over time the resistant microbes predominate (Levy 2002, Hawkey 2008). Two such environments where antibiotic selection pressure is intense are the clinical, such as hospital intensive care units (ICU) and agricultural settings related to food animal production. Hospital ICUs are an ideal environment for the emergence, proliferation and maintenance of AR bacteria. Patients receive extended treatment with the majority being administered multiple broad spectrum antibiotics (Iredell and Lipman 2005). Certain bacteria have adapted to this environment and established nosocomial strains that pose a significant challenge both for treatment and containment (Hawkey 2008). Another prime environment for

the emergence of AR bacteria is food animal production systems. Silbergeld et al. (2008) listed four reasons why the use of antimicrobials in food animal production is a significant contributor to antimicrobial resistance, 1) a large quantity of antibiotics are administered, the majority for growth promotion, 2) abundant subtherapeutic exposure of microbes to the agents both in the animals and passed with manure into the environment, 3) all major classes of antibiotics are used and 4) human exposure by those handling the livestock and through consumption of food products. There is evidence that the AR bacteria and genes that are promoted in animals exposed to antibiotics are transferred to other environments. A study focused on antibiotic resistant bacteria from archived soil samples collected from 1940 to 2008 in the Netherlands revealed a significant increase, primarily for resistance genes to β-lactamases and tetracyclines, over the period (Knapp et al. 2010). The authors attributed the rise in AR gene prevalence primarily to agricultural use of antibiotics for growth promotion from waste discarded and contaminating the soil. Further, AR genes have been detected in diverse water sources such as rivers, ponds, diary lagoons and ditch water (Pruden et al. 2006, Koike et al. 2007).

Enterococci are gram positive bacteria in the family Enterococcaceae, genus *Enterococcus* (Schleifer and Kilpper-Balz 1984). They are facultative anaerobic, lactic acid cocci and are tolerant of a wide range temperatures, pH and nutrient-poor environments (Lleo et al. 1998, Heim et al. 2002, Fisher and Phillips 2009). The primary niche of enterococci is the digestive tract of a wide range of animals including humans. *E. faecalis* and *E. faecium* are two of the most prevalent species and are of most medical and veterinary significance. Among the human gut microbiota, *E. faecalis* and *E. faecium* are the most common enterococcal species accounting for approximately 1% of the total bacteria with a concentration of 10^2-10^8 CFU/g of feces (Ogier and Serror 2008, Fisher and Phillips 2009).

Over the past few decades enterococci have emerged as the third most common nosocomial pathogens after *Escherichia coli* and *Staphylococcus aureus* (French 1998, Arias and Murray 2009). *E. faecalis* and *E. faecium* are responsible for the majority of infections with an estimated rate of 60/40% between the two species, respectively (Top 2007, Hidron 2008). Factors contributing to the significance of enterococci as nosocomial pathogens include their hardiness, intrinsic AR, acquiring resistance determinants horizontally and the presence of various putative virulence factors. Antibiotic resistance among enterococci has been observed to every major class of antibiotics, either intrinsically or acquired. Enterococci are intrinsically resistant to low concentrations of *β*-lactams, quinolones, lincosamides and aminoglycosides (Landman and Quale 1997, Ogier and Serror 2008, Top et al. 2008). Resistance is commonly acquired to *β*-lactams (methicillin, ampicillin), macrolides (erythromycin), lincosamides, tetracyclines (tetracycline, doxycycline), aminoglycosides (streptomycin, gentamicin) and streptogramins (quinupristin/dalfopristin) and increasingly to glycopeptides (vancomycin) (Kak and Chow 2002, Arias and Murray 2008). A number of virulence factors are associated with clinically significant enterococcal strains that aid in avoidance of host immune responses and/or breakdown of host tissue. The best studied of these are gelatinase (Gel), cytolysin (Cyl), aggregation substance (AS) and enterococcal surface protein (Esp) (Gillmore et al. 2002, Hancock and Gillmore 2006, Ogier and Serror 2008, Upadhyaya et al. 2009). Gelatinase is a metalloprotease that causes degradation of host tissues and is associated with formation of biofilm (Hancock and Gillmore 2006, Mohamed and Huang 2007). Cytolysin is a bacterial toxin that also displays hemolytic activity and is associated with enterococci causing bacteremia. Aggregation substance is a surface-localized protein expressed among *E. faecalis* and is involved in pheromone responsive plasmid conjugation. Aggregation substance is also suspected to play a

role in binding to eukaryotic cells and is positively associated with urinary tract infections (UTI). Additionally, AS plays a putative role in binding and traversing the gut barrier (Waters et al. 2003). Enterococcal surface protein is another surface protein which plays a role in biofilm formation and UTI (Gillmore et al. 2002, Hancock and Gillmore 2006, Mohamed and Huang 2007). A synergistic effect of virulence involving endocarditis has been shown among enterococci that contain both AS and Cyl (Chow et al. 1993). Both of these virulence factors are frequently transferred horizontally among *E. faecalis* on pheromone responsive plasmids (Hegstad et al. 2010). Additionally, Esp, AS and Cyl are frequently found on pathogenicity islands, a large chromosomal region that encodes virulence (Ogier and Serror 2008, Upadhyaya et al. 2009).

In addition to being opportunistic pathogens, enterococci are also considered reservoirs of antibiotic resistance genes, thus playing an important role in AR gene ecology. Horizontal gene transfer (HGT) has been identified as a way that genes are conferred both intra and interspecifically and can bridge ecological barriers that the bacteria themselves may not be capable of (Kotzamanidis et al. 2009). Horizontal gene transfer involving enterococci has been observed in the digestive tract of humans (Lester et al. 2006), mice (Dahl et al. 2007, Moubareck et al. 2003), rats (Jacobsen et al. 2007) and house flies (Akhtar et al. 2009) and in wastewater during treatment (Marcinek et al. 1998). A primary concern of HGT is that AR genes can spread to other enterococci or more pathogenic bacteria such as *Staphylococcus aureus* (Huycke *et al.* 1998, Sung et al. 2007, Fisher and Phillips 2009).

The house fly, *Musca domestica* (L.) is the most common fly species in the family Muscidae and is distributed worldwide. The house fly is a significant nuisance pest due to the high populations and its synanthropic nature. Additionally, the fly is recognized as an efficient

mechanical vector of a number of parasites/pathogens including protozoans, viruses, fungi and bacteria (Greenberg 1965, Graczyk et al. 2001). A number of house fly attributes contribute to its ability to function as a mechanical vector, namely its feeding habits, abundance and close association with humans, the nature of larval developmental sites and dispersal habits. The house fly ingests microbes associated with its food sources and oviposition sites, which can reside transiently in the fly digestive tract, proliferate and can be disseminated through feeding and defecation (Sasaki et al. 2000, Kobayashi et al. 2002). Kobayashi et al. (1999) proposed the term "bioenhanced transmission" to describe this phenomenon, which is more than simple mechanical transmission.

A few studies have specifically considered the association of *M. domestica* and enterococci. Graham et al. (2009) isolated and characterized enterococci from poultry litter at three operations and from house flies both onsite and up to 3.2 km away in a tristate region (Delaware, Maryland and Virginia). The genotypic and phenotypic AR profiles of enterococci from litter and flies matched well, suggesting that the flies play a role in the ecology and dissemination of enterococci and associated resistance genes at these operations (Graham et al. 2009). Enterococci were also characterized from house flies, German cockroaches and swine feces at two swine operations in Kansas and North Carolina (Ahmad et al. 2011). Ninety four percent of house flies were positive for enterococci with *E. faecalis* as the most abundant species recovered from flies. Both house flies and swine manure had similar phenotypic and genotypic AR profiles as well as AR genes and mobile genetic elements. Genotyping of *E. faecalis* and *E. faecium* isolates with pulsed-field gel electrophoresis (PFGE) revealed clonal matches among the bacteria from feces, house flies and roaches. Another study assessed the AR profiles of flies captured in rural and urban environments as part of an effort to assess fly migration up to 124 km

to and from Manhattan, KS (Chakrabarti et al. 2010). Over 90% of the flies were positive for enterococci and the AR prevalence among the bacteria was highest among house flies captured at cattle feedlots followed by dairies and urban sites such as restaurants. The authors attributed the feedlots as the primary source of AR enterococci. Macovei and Zurek (2006) sampled the digestive tracts of *M. domestica* for enterococci at five fast-food restaurants in Northeastern Kansas and found AR enterococci common among the *M. domestica*. Ninety seven percent of the flies were positive for enterococci with a mean CFU of 3.1×10^3/fly. Two hundred and five of the enterococci isolates cultured were characterized. *E. faecalis* was the most abundant species (88.2%) and AR phenotypic tests revealed resistance to tetracycline, erythromycin, streptomycin, ciprofloxacin and kanamycin. Further, several virulence genes were identified among the isolates including *gelE*, *asa1*, *esp* and *cylA* (Macovei and Zurek 2006). A follow-up study found food from the same restaurants was commonly contaminated with AR enterococci (Macovei and Zurek 2007). Three ready-to-eat food items were sampled in summer and winter. Overall concentration of enterococci throughout the year averaged 10^3 CFU/g with greater prevalence during the summer, which correlates with higher house fly activity, thus indirectly implicating the fly as at least a partial source of contamination (Macovei and Zurek 2007). A lab-based study revealed that house flies readily contaminate ready-to-eat food with enterococci (Macovei et al. 2008). The authors observed that as few as five flies in as little as 30 minutes (the fewest flies and shortest time tested) would result in an average of 3.1×10^3 enterococci deposited on the food.

These studies showed either direct or indirect evidence that an agricultural source (poultry, swine and cattle operations) were the source of the AR enterococci and that house flies were a likely vector. Indeed, the interface of microbes/pathogens of food production animal

origin and house flies has been well established (Rosef and Kapperud 1983, Buma et al. 1999, Iwasa et al. 1999, Mian et al. 2002, Graham et al. 2009, Chakrabarti et al. 2010) and due to the abundant access house flies have to numerous sources of animal waste, this is likely the primary source of enterococci, particularly in rural areas.

Another potential source of AR enterococci in *M. domestica* involves a human/clinical source, specifically that of a clinical origin where there is considerable antibiotic pressure. Though it is unlikely that flies gain significant access to hospitals or health clinics directly or indirectly via medical waste in developed countries, sewage waste from these sources is a potential point source at municipal wastewater treatment facilities. This may be a significant source of microbes for house flies, particularly in urban settings.

A 2007 report from the North East Biosolids and Residuals Association (NEBRA) estimated that there were approximately 17,000 operating wastewater treatment facilities (WWTF) in the US (NEBRA 2007). Wastewater treatment facilities serve as the principal consolidation point for human waste in industrialized societies and play a significant role in the ecology of numerous microbes. Many of the bacteria shed in human waste can potentially end up at the treatment facility including those harboring antibiotic resistance. Antibiotic resistant bacteria have long been observed in wastewater raising the concern that the bacteria and associated AR determinants could be passed to other microbes and potentially released to the environment through plant effluent and biosolids (Sturtevant and Feary 1969, Grabow and Prozesky 1973). Waste received from environments under significant antibiotic pressure such as hospitals consistently contains a greater percentage of single and multiple drug resistant bacteria (Grabow and Prozesky 1973, Guardabassi et al. 1998, Harwood et al. 2001, Blanch et al. 2003, Reinthaler et al. 2003, Kotzamanidis et al. 2009) and AR enterococci are commonly recovered

from sewage (Harwood et al. 2001, Blanch et al. 2003, Martins da Costa et al. 2006, Poole et al. 2005, Ahmed et al. 2008, Beier et al. 2008, Nagulapally et al. 2009, Kotzamanidis et al. 2009).

Wastewater treatment operations primarily receive human waste including hospital sewage where high prevalence of AR bacteria is common. Antibiotic resistant enterococci are frequently present in sewage and though they are reduced during waste treatment, they are not completely eliminated (Iversen et al. 2004, Ferreira da Silva et al. 2006, Martins da Costa et al. 2006, Nagulapally et al. 2009). House flies and other filth flies often have direct and unhindered access to many steps in the waste processing flow, including bar screening, aeration tanks, clarifiers, sludge digesters and biosolid storage. House flies therefore can acquire bacteria associated with waste both externally on the body and internally in the digestive tract. If the flies then disperse from the WWTF they can carry and possibly disseminate the microbes to other areas, with a potential impact on human health. This route of bacteria dispersal from WWTF is the focus of this study.

The objective and hypothesis for this study were presented in Chapter 1 as Objective 1:

Objective 1: Isolate and characterize enterococci from four wastewater treatment facilities (house flies and sludge/biosolids) and house flies in the surrounding residential environment.

Hypothesis: House flies acquire and disseminate antibiotic resistant enterococci from wastewater treatment facility to the surrounding environment.

2.3 MATERIALS AND METHODS

2.3.1 Study duration

Samples of sludge and flies were collected during 25 visits to four wastewater treatment facilities (WWTF) from August 2008 to July 2010. Six visits were conducted in 2008 from 14 Aug – 1 Oct; during 2009, 15 visits from 2 Jun – 11 Aug; and in 2010, samples were collected

exclusively from Site 1 WWTF and locations up to 2 km away during four visits from 7 Jun – 12 Jul.

2.3.2 Study sites

Four wastewater treatment facilities (Sites 1-4) were sampled. The operations utilized the activated sludge technique for processing liquid waste with forced aeration during primary treatment (Horan 1990, Cheremisinoff 1996). Sludge was sampled from each of the WWTF to isolate enterococci. Criteria for sludge collection at the facilities involved identifying locations that offered access to house flies and where flies were visually most abundant. Therefore, the focus for sampling solid waste was not to ensure uniformity among the wastewater facilities, but to sample waste that flies were contacting and presumably acquiring microbes from. The term sludge can represent three specific sources of solids, bar screening waste, insoluble grit, or treated biosolids, which is the end product of sludge digestion (Horan 1990, Cheremisinoff 1996, Arthurson 2008). Details are provided below regarding sludge sources sampled at each WWTF. Among the potential sources of AR enterococci, hospital ICUs are likely a primary contributor. Therefore, the presence/absence and relative size of hospitals/ICUs the WWTF received waste from is noted below for each site.

Site 1. Site 1 was one of two WWTF for a northeast Kansas community of 21,000. Site 1 received approximately 2.8 million liters of waste daily from two sources; residential sewage (95,000 liters) and industrial waste from a nearby commercial sausage plant (1.9 million liters). The residential sewage at Site 1 did not include any waste from hospitals or other known clinical sources. The sausage plant was a 17,000 m^2 facility that produced ready-to-eat cooked sausage. Waste from this facility arrived through a dedicated line and was initially kept separate from sewage. The solids of the industrial waste (primarily meat remains) were separated and

temporarily stored in open containers (garbage dumpsters) and were removed weekly by a contractor. Following the separation of solid waste from the sausage plant influent, the liquids were mixed with sewage for combined treatment.   The stored meat waste solids were the focus of sludge and house fly sampling at Site 1 due to the abundance of flies at this source.   Human sewage sludge was stored in a closed container, which limited fly access and therefore was not sampled at this site.

Site 2. Site 2 was the second WWTF for the same community of 21,000 and processed approximately 6.8 million liters of waste daily including influent from a small community hospital with a four-bed ICU. Sludge and biosolids were digested/stored in closed containers that limited fly access. The majority of fly activity at site 2 was at the initial bar screen and the grit removal stations. All solid/sludge samples as well as house flies from this facility were collected at these points.

Site 3. Site 3 served a community of 53,000 and received 20.8 million liters of waste daily including waste from a medium size hospital with an eight-bed ICU and four-bed intermediate ICU. Waste treatment at Site 3 did not include a primary clarifier. The waste flow was directed to an aeration basin following initial bar screening and grit removal. The only clarifier in this system was located following aeration. Flies and solids were sampled among three locations, the bar screen, grit removal station and on the margins of the clarifier where floating scum was removed and consolidated.

Site 4. Site 4 received 1.7 million liters of waste daily from a community of 4,400. The community was served by a small medical facility of 25 beds with no ICU. Sludge was processed using aerobic digestion (Arthurson 2008) for approximately 20 days, after which it was dried by use of a belt press and stored in an open field onsite. Sludge/solids from site 4 were

collected at two primary locations, the bar screen and from treated biosolids that were stored onsite.

All study sites were sampled equally during 2008 to estimate the overall fly activity, enterococcal diversity and antibiotic resistance/virulence. Based on the results of Site 1 including the antibiotic resistance profiles of enterococci, the abundance of flies and the unique source of industrial waste received, this site was the focus of more sampling during 2009 and exclusively during 2010. The goal for obtaining isolates to characterize among the four sites was: Site 1), minimum 100 *E. faecalis*/*E. faecium* from each of three sources, sludge, house flies caught onsite and house flies caught offsite and for Sites 2,3,4), combined minimum 100 *E. faecalis*/*E. faecium* characterized from each of two sources, sludge samples and house flies onsite.

2.3.3 Locations sampled near Site 1 WWTF

House flies were sampled for enterococci at three locations near Site 1. Sites were selected based on the greatest potential for human/fly contact; a recreational vehicle (RV) park, a fast food restaurant and at an apartment complex (Fig. 2.1) No significant HF larval habitats or feeding sites (e.g. feed lots, swine or poultry operations) were noted during visual surveys of the area within a few kilometers of Site 1. The significance of this observation is that Site 1 WWTF was likely the most significant feeding site for HF in the area, which could have increased the probability of collecting flies that had contacted and fed on the waste at Site 1 from the offsite locations described below.

RV Park. House flies were collected at a small RV park located approximately 0.7 km from Site 1 (approximately 30 residents) during 2010. Flies were collected around the trash dumpster.

Restaurant. House flies were collected from a fast-food restaurant located approximately 1 km from Site 1 during 2009. Flies were collected inside the facility and outside near the trash dumpster.

Apartments. House flies were collected from a 600 unit apartment complex with approximately 1200 tenants located 1.5-2 km from Site 1. Flies were captured in and around the trash dumpsters during 2009 and 2010.

2.3.4 Isolation of enterococci from sludge and house flies

House flies were collected with a sweep net, transferred to a self-sealing plastic bag and placed on ice for transport to the laboratory. Sludge samples were placed into sterile containers and set on ice. All samples were processed immediately after arrival to the laboratory. One gram of sludge was homogenized in 10 ml of sterile phosphate buffered saline (PBS). House flies were surface sterilized with 10% sodium hypochlorite (bleach) and 70% ethanol (1 min in each) (Zurek et al. 2000) and homogenized individually in 1 ml of sterile PBS. All sludge and fly samples were 1/10 serially diluted and drop plated on m-Enterococcus (m-Ent) agar (Difco, Franklin Lakes, NJ). Plates were incubated at 37ºC for 48 h upon which the concentration of enterococci was determined by counting colony forming units (CFU) and up to five dark purple colored colonies, presumptive of *Enterococcus* genus, were picked from each sample for characterization. Criteria for selection of isolates from m-Ent agar plates during 2008 was to maximize the diversity of colonies present in order to better assess the enterococcal species diversity. This involved sampling each morphologically distinct colony present on the plates. For the 2009 and 2010 seasons, colonies were selected that were suspected to be *E. faecalis* or *E. faecium* based on morphology as these were the only two species characterized during these years.   Each colony was streaked on Trypticase Soy Broth Agar (TSBA) (Difco, Franklin

Lakes, NJ) to obtain a pure culture. To confirm the *Enterococcus* genus, the esculin hydrolysis test (Qadri et al. 1987) was conducted by culturing isolates in Enterococcosel™ broth (Difco, Franklin Lakes, NJ) for 4 h at 44ºC. All positive isolates were transferred into TSBA (0.3% agar) in 2.0 ml vials and stocked at 10°C. Sludge and house fly homogenates during 2009 and 2010 were screened for vancomycin resistant enterococci. m-Ent agar plates with 16 mg/L vancomycin were inoculated with sludge and house fly homogenates. All colonies found on these plates after 48 h incubation at 37ºC were tested with the esculin hydryolysis test to verify identification to genus *Enterococcus*.

2.3.5 Identification of *Enterococcus* spp.

   E. faecalis and *E. faecium* were identified by multiplex PCR with primers for D-alanine–D-alanine ligase (*ddl*) specific for each species (Kariyama et al. 2000, Elsayed et al. 2001). *Enterococcus faecalis* V583 and *E. faecium* ATCC 19434 were used as positive controls. Isolates not identified as *E. faecalis* or *E. faecium* during the 2008 season were identified by sequencing the superoxide dismutase gene (*sodA)* (Poyart et al. 2000). The gene was amplified by PCR and purified with the DNA Clean & Concentrator™-5 Kit (Zymo Research, Irvine, CA). DNA quality was confirmed by micro-volume spectrophotometer, NanoDrop 2000 (Thermo Scientific, Wilmington, DE). Samples were then sequenced by the Genome Core Facility (University of California, Riverside) using the same primers and results were used to identify species by use of BLAST search in the NCBI GenBank database (http://blast.ncbi.nlm.nih.gov). For 2009 and 2010, only *E. faecalis* and *E. faecium* were identified (PCR) and characterized due to the predominance of these two species (Fig. 2.2) and their clinical significance.

2.3.6 Antibiotic resistance phenotype test by disk diffusion or agar dilution

All identified isolates were assessed for antibiotic susceptibility. Screening for nine antibiotics was done by disk diffusion technique on Mueller-Hinton Agar (Difco, Franklin Lakes, NJ). Each isolate was initially streaked to TSBA and incubated for 24 h at 37ºC to obtain fresh colonies followed by transfer to 1 ml of sterile PBS to 0.5 McFarland. 100 µl of the suspended isolate was drop-plated to Mueller-Hinton agar; up to nine antibiotic discs were placed on the plate and incubated for 24 h at 37ºC. The zone of isolate growth/inhibition around the respective disc was measured to determine susceptibility/resistance based on breakpoints included below. The nine antibiotics were doxycycline (30 µg, resistance breakpoint ≤ 12 mm), gentamicin (120 µg, resistance breakpoint ≤ 6 mm), erythromycin (15 µg, resistance breakpoint ≤ 13 mm), ampicillin (10 µg, resistance breakpoint ≤ 13 mm), quinupristin/dalfopristin (*E. faecium* only) (15 µg, resistance breakpoint ≤ 15 mm), ciprofloxacin (5 µg, resistance breakpoint ≤ 15 mm), vancomycin (30 µg, resistance breakpoint ≤ 14 mm), nitrofuratoin (300 µg, resistance breakpoint ≤ 14 mm), and tigecycline (15 µg, resistance breakpoint ≤ 14 mm).

The agar dilution technique was used to determine resistance to streptomycin (2 g/L), tetracycline (16 mg/L) and linezolid (8 mg/L) added to Mueller-Hinton agar. Isolates were spotted onto each plate and incubated for 24 h at 37ºC. If isolate growth was observed it was considered resistant. Positive controls were *E. faecalis* OGIRF for tetracycline, *E. faecalis* OG1SSP for streptomycin and *E. faecalis* 41-31 (wild isolate) for linezolid. The Clinical and Laboratory Standards Institute protocols were used as standards for these procedures (Clinical and Laboratory Standards Institute, 2005).

2.3.7 PCR for virulence genes: *cylA*, *gelE*, *esp* and *asa1*

E. faecalis and *E. faecium* isolates were screened with multiplex PCR for the following virulence genes: *gelE* (gelatinase activity), *cylA* (cytolysin, hemolytic activity), *esp* (enterococcal

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surface protein) and *asa1* (aggregation substance) (*E. faecalis* only) (Vankerckhoven et al. 2004). *E. faecalis* MMH 594 was used as a positive control for all genes. *E. faecium* isolates were not screened for *asa1* as this species does not possess the aggregation gene.

2.3.8 Phenotypic tests for virulence factors, gelatinase, cytolysin and aggregation substance

Gelatinase activity was determined on Todd Hewitt Broth (THB) (Difco, Franklin Lakes, NJ) agar plates with 1.5% dry skim milk powder (Macovei et al. 2009). Isolates were streaked to TSB plates and grown overnight at 37ºC and then spotted onto the THB/skim milk plates. Following incubation at 37ºC for 24 h, plates were examined for a clearance zone to assess gelatinase activity. Isolates were characterized as either gelatinase negative (no clearance), weak gelatinase (some clearance) or strong gelatinase (wide area of clearance) (Macovei et al. 2009).

Cytolysin gene expression was evaluated by streaking the isolates on Columbia blood agar (Difco, Franklin Lakes, NJ) with 5% human blood and incubated at 37ºC for 24 h. Hemolytic activity was assessed by measuring the clearing zone around colonies. A large clearance zone was determined as *β*-hemolysis and recorded positive for cytolysin gene expression. *E. faecalis* OG1X:pAM1 was used as a positive control for *β*-hemolysis.

Enterococcal aggregation substance was screened phenotypically for all Site 1 *E. faecalis* strains by the clumping assay (Dunny et al. 1978). *E. faecalis* JH2-2 was used for cCF10 peptide formation. THB was used to grow *E. faecalis* JH2-2 and incubated at 37°C for 18 h. The pheromone peptide in the supernatant was collected by centrifugiation (10,000 rcf for 10 min) and then sterilized by autoclaving for 15 min. *E. faecalis* isolates were cultured in THB broth for 18 h at 37ºC, then 1 ml *E. faecalis* JH2-2 supernatant was added to each culture and incubated at 37°C overnight in a shaker incubator. After the incubation period, isolates were considered positive if clumping or aggregation of cells was visually observed. *E. faecalis* OG1RF (pCF10)

was used as positive control and *E. faecalis* OG1SSP a negative control with every batch of isolates (Dunny et al. 1978).

2.3.9 Conjugation assays

Conjugation assays were performed for multiple antibiotic resistant *E. faecalis* (8 isolates) and *E. faecium* (2 isolates) to test for horizontal transfer of resistance determinants. Selection of antibiotics used in brain heart infusion broth (BHI) agar plates was done based on the resistance pattern of the donor and recipient strains. Concentrations of antibiotics added to BHI agar are listed in parentheses following mention of the specific antibiotic.

For transconjugation of *E. faecalis*, the recipient strain for transfer of gentamicin (500 mg/L), tetracycline (16 mg/L), doxycycline (0.5 mg/L) and erythromycin (32 mg/L) was *E. faecalis* OG1SSP using marker antibiotic spectinomycin (250 mg/L). The recipient for streptomycin (2 g/L) was a wild isolate *E. faecalis* 41-31 with linezolid marker (8 mg/L).

Two isolates of *E. faecium* were tested for resistance transfer. The recipient for transfer of erythromycin (32 mg/L), ciprofloxacin (enrofloxacin, 8 mg/L), streptomycin (2 g/L) and tetracycline (16 mg/L) was wild isolate *E. faecium* 45-26 using linezolid marker (8 mg/L). For transfer of ampicillin (64 mg/L), the recipient *E. faecium* ATCC 51559 was used with vancomycin (16 mg/L) as the marker.

Conjugation frequencies were determined by filter mating and broth mating assays using donor and recipient cultures grown overnight at 37ºC in BHI broth as described previously (Dunny et al. 1979, Tendolkar et al. 2006). Broth mating was performed by mixing 500 μl of the recipient strain to 50 μl of donor (1:10 donor: recipient) in 4.5 ml of fresh BHI broth and incubating at 37°C for 4 h at 100 rpm. Following incubation, the donor/recipient culture was 1/10 serially diluted and spread plated on BHI agar plates containing appropriate selective

antibiotics. One plate contained the antibiotic to which the donor strain was resistant (for determining donor concentration), another contained the marker antibiotic of the recipient strain (for determining the recipient concentration), and the final plate contained both antibiotics (for determining transconjugants). Colonies were counted after 48 h of incubation at 37°C. Any colonies present on plates containing both antibiotics (donor resistance and recipient marker agents) were counted as transconjugants. Transfer frequency was determined by number of transconjugants per donor cell.

For filter mating, 500 μl of the donor and 4.5 ml of the recipient (1:10 donor: recipient) from BHI broth was passed through autoclaved 0.22 μm pore size nitrocellulose membrane filter (Whatman International Ltd., Germany). The membrane filter was placed on a BHI agar plate and incubated at 37°C for 16 h. Following incubation, cells from the filter were harvested by suspending the filter in 1 ml sterile PBS. The suspension was 1/10 serially diluted and spread plated on BHI agar plates containing appropriate selective antibiotics as described above. Colonies were counted after 48 h of incubation at 37°C and identified/calculated as described for broth mating.

2.3.10 Pulsed-field gel electrophoresis (PFGE)

PFGE was performed to genotype isolates from WWTF sludge and house flies following the protocol of Amachawadi et al. (2010) with the following modifications:

Restriction enzyme digestion. One third of the 1.6% SeaKem Gold® agarose (FMC™) plug was cut and washed prior to restriction digestion in 10 μl bufferA and 90μl autoclaved distilled water for 10 min at 25°C. Cleavage of the DNA was achieved with 0.5 μl *ApaI* in 10 μl of buffer and 89.5 μl autoclaved distilled water and incubated in a waterbath at 37°C for 4 h.

Electrophoresis settings. The CHEF-MAPPER ^{TM} (BIORAD) was used for electrophoresis in 0.5X TBE as described by Murray et al. (1990). Settings included: Run time 21 h, temperature 14°C, initial switch time 1 second, final switch time 20 seconds, included angle 120, voltage 6 V/cm.

The profiles of the isolates were analyzed with BioNumerics software (Applied Maths Inc., Austin, TX). Dendograms were created using Dice similarity coefficient (1% optimization) and unweighted pair group method with arithmetic means (UPGMA) clustering analysis with 1.5% band position tolerance. *Enterococcus faecium* ATCC 19454 was used as a reference strain.

2.3.11 Statistical analysis

Statistical analysis was performed to evaluate differences of antibiotic resistance prevalence and virulence genotypic profiles among *E. faecalis* based on wastewater treatment site (Sites 1-4) and isolate source (sludge, onsite house flies, offsite house flies). This was done only for *E. faecalis* as *E. faecium* were not isolated in sufficient numbers for adequate comparison. There was a cluster effect among individual isolates due to multiple isolates originating from the same house fly or sludge sample, i.e. the isolates from the same sample were not independent. As such, a mixed-effect logistic regression model was used to test for differences among *E. faecalis* from the four wastewater treatment sites (Sites 1-4) and three sources (sludge, house flies onsite, house flies offsite) accounting for the cluster effect by sample (Vermunt 2005, Dohoo et al. 2009).

The antibiotic resistance profiles among *E. faecalis* from Site 1 were compared to the combined results from Sites 2, 3, 4 due to the fundamental differences in the waste received (industrial meat waste at Site 1 versus sewage at Sites 2,3,4). A mixed-effect logistic regression

model used site (Site 1 vs. Sites 2,3,4) and isolate source (sludge, onsite houseflies) as fixed effects, variance components due to repeated observations within site and flies as random effects, and isolate resistance/susceptibility to tetracycline, doxycycline, streptomycin, gentamicin, and erythromycin as the response variables (Dohoo et al. 2009).

Antibiotic resistance prevalence among *E. faecalis* at Site 1 was further analyzed by adding a third source, offsite house flies, to the two already mentioned. The regression model in this test used source (sludge, onsite house flies, offsite house flies) as the fixed effect, variance component due to repeated observations within flies as the random effect, and isolate resistance/susceptibility to tetracycline, doxycycline, streptomycin, gentamicin, and erythromycin as the response variables.

The virulence genotype of *E. faecalis* at Site 1 was also analyzed using a mixed-effect logistic regression with source (sludge, onsite house flies, offsite house flies) as the fixed effect and presence/absence of *gelE*, *asa1*, *esp* and *cylA* as the random effect (Dohoo et al. 2009).

Significance level for all analyses was $P < 0.05$.

2.4 RESULTS

2.4.1 Prevalence, quantification and identification of enterococci

During the first season of the study (2008), all enterococcal isolates chosen from selective media plates were identified to species in order to appraise the diversity in this environment. Two hundred twenty five enterococci were identified consisting of 11 species, of which the majority (76.9%) were *E. faecalis* (60.4%) and *E. faecium* (16.4%) (Fig. 2.2). Other species identified were *E. flavescens*, *E. casseliflavus*, *E. gallinarum*, *E. malodoratus*, *E. sulfureus*, *E. durans*, *E. avium*, *E. moraviensis* and *E. hirae* (Fig. 2.2). Six species were isolated from sludge samples and eight from house flies. From Site 1, three species were isolated from 18 sludge

isolates, *E. faecalis* (11/18, 61.1%), *E. flavescens* (5/18, 27.8%) and *E. casseliflavus* (2/18, 11.1%) and from house flies, *E. faecalis* was most abundant (43/59, 72.9%) followed by *E. malodoratus* (10/59, 16.9%) (Fig. 2.2). Among Sites 2, 3, and 4, 36 isolates from 14 sludge samples were identified consisting of three species, 17 *E. faecalis* (47.2%), 17 *E. faecium* (47.2%) and one *E. avium* (2.8%) and one *E. hirae* (2.8%). Among 51 house flies from Sites 2, 3, and 4, 112 enterococci were identified consisting of seven species. Of these, the majority (83/112, 74.1%) were *E. faecalis* (65/112, 58.0%) and *E. faecium* (18/112, 16.1%) (Fig. 2.2).

A total of 89 sludge samples were analyzed for enterococci, of which 84 (94.4%) were positive for enterococci (Table 2.1). The mean concentration of enterococci was $2.3 \pm 0.8 \times 10^6$ and ranged from $2.5 \pm 0.6 \times 10^4$ (Site 4) to $4.9 \pm 1.5 \times 10^6$ (Site 1) (Table 2.1). Two hundred six isolates were characterized from these samples, of which 161 (78.2%) were *E. faecalis* and 45 (21.8%) were *E. faecium*. Further details regarding the specific number of isolates collected and concentrations from each location are included in Table 2.1.

A total of 276 house flies (HF) were collected during the study, of which 181 (65.6%) were positive for enterococci (Table 2.2). The mean concentration of enterococci was $8.9 \pm 3.4 \times 10^3$ and ranged from $3.7 \pm 1.8 \times 10^3$ (Site 2) to $1.5 \pm 1.0 \times 10^4$ (HF offsite of Site 1) (Table 2.2). Three hundred fifty six isolates (*E. faecalis* or *E. faecium*) were selected for characterization, of which 296 (83.1%) were *E. faecalis* and 60 (16.8%) were *E. faecium*. Further details regarding the specific number of isolates collected and concentrations from each location are included in Table 2.2.

Of the locations sampled offsite of Site 1 the following isolates were characterized from each location. RV Park; 24 *E. faecalis* isolates and 1 *E. faecium* were characterized from 8 house flies. Restaurant; 10 *E. faecalis* and 8 *E. faecium* were characterized from 9 house flies

within the restaurant and 14 *E. faecalis* and 14 *E. faecium* were characterized from 6 house flies outside. Apartments; 48 *E. faecalis* and 9 *E. faecium* were characterized from 16 house flies.

All Sludge and house fly homogenates were screened for the presence of vancomycin resistant enterococci during 2009 and 2010 by drop plating on enterococci-selective media with 16 mg/L vancomycin added and incubating for 48 h at 37ºC. Results were negative; no vancomycin resistant enterococci were isolated during the study.

2.4.2 Phenotypic antibiotic resistance of *E. faecalis*

E. faecalis isolates were tested for resistance/susceptibility to 11 antibiotics. Isolates were most commonly resistant to one or more of five antibiotics, tetracycline, doxycycline, streptomycin, gentamicin and erythromycin with tetracycline resistance the most common (Fig. 2.3, 2.4, Table 2.3). None of *E. faecalis* were resistant to ampicillin, vancomycin, linezolid or tigecycline (Figs. 2.3, 2.4). *E. faecalis* from Site 1 WWTF expressed significantly higher resistance than those from Sites 2, 3 and 4 (erythromycin and streptomycin, $P < 0.01$; tetracycline, doxycycline and gentamicin, $P < 0.0001$) (Fig. 2.3).

Site 1. Of 88 isolates from 24 sludge samples, 85.2% were resistant to tetracycline followed by doxycycline (76.1%), erythromycin and gentamicin (both 52.3%), and streptomycin (35.2%) (Fig. 2.3, 2.4, Table 2.3). Among 120 *E. faecalis* from 44 house flies collected at Site1 (onsite HF), 71.7% were resistant to tetracycline followed by doxycycline (54.2%), gentamicin (40.8%), erythromycin (39.2%), streptomycin (25.0%) and nitrofurantoin (1.7%).   The resistance prevalence was not significantly different for sludge and onsite house flies for tetracycline, erythromycin, streptomycin and gentamicin and significantly different for doxycycline ($P < 0.05$) (Fig. 2.3, 2.4).   Isolates from house flies captured offsite of Site 1 (RV park, restaurant and apartment complex 0.7 - 2 km away) expressed resistance to the same agents

as those from the sludge but the overall prevalence was significantly lower for tetracycline ($P < 0.0001$), doxycycline ($P < 0.0001$), erythromycin ($P < 0.0001$) gentamicin ($P < 0.0001$) and not significantly different for streptomycin (Fig. 2.4). Of 98 *E. faecalis* from 31 house flies, 25.5% were resistant to tetracycline followed by doxycycline (16.3%), erythromycin and gentamicin (both 8.2%) and streptomycin (6.1%) (Fig. 2.4, Table 2.3). No apparent trend with regard to enterococci resistant prevalence was observed among the three locations sampled, i.e. prevalence did not diminish with increasing distance from Site 1.

The pattern of multiple antibiotic resistances from Site 1 corresponded best among *E. faecalis* isolates from sludge and onsite house flies and less well among isolates from offsite house flies (Table 2.4). For example, resistance to the combination of tetracycline, doxycycline and gentamicin was observed among sludge, onsite HF and offsite HF at 15.9%, 12.5% and 1.0%, respectively and resistance to the five antibiotics tetracycline, doxycycline, erythromycin, streptomycin and gentamicin occurred at 25.0%, 20.0% and 1.0%, respectively (Table 2.4).

Sites 2, 3. 4. Antibiotic resistance profiles are presented combined for these three sites in Figure 2.3. Among the 73 *E. faecalis* isolates from 29 sludge samples, the greatest percentage were resistant to tetracycline (39.7%) followed by erythromycin (27.4%), doxycycline (15.1%) streptomycin (11.0%), gentamicin (2.7%) and ciprofloxacin (1.4%). Among 78 isolates from 42 house flies, the majority were resistant to tetracycline (56.4%) followed by erythromycin (14.1%), doxycycline (17.9%), streptomycin (1.3%) and gentamicin (2.6%). Resistance profiles were compared between Site 1 *E. faecalis* and the combined patterns of Sites 2,3,4, which provided a contrast of *E. faecalis* from Site 1 industrial meat waste versus human sewage at Sites 2,3,4. The prevalence of resistance between the two (Site 1 versus Sites 2,3,4) was significantly

different for tetracycline ($P < 0.0001$), erythromycin ($P < 0.01$), streptomycin ($P < 0.01$) and gentamycin ($P < 0.0001$) and not different for doxycycline.

Site 2. Among sludge isolates, 35.5% and 47.4% of house fly isolates were resistant to tetracycline (Table 2.3). Erythromycin resistance was observed among 22.6% of sludge isolates and 26.3% of house fly isolates. Doxycycline resistance was observed among 3.2% of sludge isolates and 15.8% of house fly isolates. Additionally, 3.2% of sludge isolates were resistant to gentamicin and 5.3% from house flies. No *E. faecalis* isolates from Site 2 were resistant to streptomycin (Table 2.3).

Site 3. Among 29 sludge isolates and 26 house fly isolates, 31.0% from sludge and 50.0% from house flies were resistant to tetracycline (Table 2.3). Erythromycin resistance was observed among 13.8% of sludge isolates and 23.1% of house fly isolates. Doxycycline resistance was observed among 6.9% of sludge isolates and 30.8% of house fly isolates (Table 2.3).

Site 4. Among 13 sludge isolates and 33 house fly isolates, 76.9% of sludge and 6.1% of house fly isolates were resistant to tetracycline (Table 2.3). Erythromycin resistance was observed among 69.2% of sludge isolates and none were resistant from house flies. Doxycycline resistance was observed among 61.5% of sludge isolates and 9.1% of house fly isolates. Streptomycin resistance was observed among 61.5% of sludge and 3.0% of house fly isolates. Gentamycin resistance was observed among 7.7% sludge and 3.0% of house fly isolates. Ciprofloxacin resistance was observed among 7.7% of sludge isolates and 3.0% of house fly isolates (Table 2.3).

2.4.3 Phenotypic antibiotic resistance of *E. faecium*

  E. faecium isolates were tested for susceptibility/resistance to 12 antibiotics. This species was less frequently isolated from sludge and flies than *E. faecalis*. From Site 1, 12 *E. faecium* isolates from 4 sludge samples, 4 from 4 house flies onsite and 32 from 15 house flies offsite were characterized (Tables 2.1, 2.2). From Site 2, 6 isolates from 5 sludge samples and 6 isolates from 6 house flies were characterized. From Site 3, 8 isolates from 5 sludge samples and 8 isolates from 5 house flies were characterized. From Site 4, 19 isolates from 9 sludge samples and 10 isolates from 6 house flies were characterized (Tables 2.1, 2.2).

  Site 1. Six of 12 *E. faecium* (50.0%) from four sludge samples were resistant to erythromycin with no other resistances observed (Fig. 2.5, 2.6, Table 2.5). Among onsite house fly isolates, 1 of 4 (25.0%) was resistant to both tetracycline and ciprofloxacin (Fig. 2.5, 2.6, Table 2.5). Among offsite house fly isolates, the greatest number of *E. faecium* (32) were recovered and characterized from 16 flies. Of those, 40.1% were resistant to tetracycline, 28.1% to ciprofloxacin, 6.3% to ampicillin, 6.3% to streptomycin, 3.1% to nitrofurantoin and 3.1% to doxycyline (Fig. 2.6, Table 2.5).

  Sites 2, 3, 4. Antibiotic resistance profiles are presented combined for these three sites in Figure 2.5. Among the 33 *E. faecalis* isolates from 19 sludge samples, the greatest percentage were resistant to erythromycin (21.2%) followed by tetracycline (9.1%) and doxycycline, quinupristin/dalfopristin and nitrofurantion each at 6.1% (Fig. 2.5). Among 24 isolates from 17 house flies, the greatest percentage were resistant to tetracycline (37.5%) followed by erythromycin (16.7%), ampicillin and ciprofloxacin both at 4.2% (Fig. 2.5).

  Site 2. Among six *E. faecium* from five sludge samples, 16.7% were resistant to tetracycline, doxycycline, erythromycin and nitrofurantoin. Of the six isolates from six house flies, 66.7% were resistant to tetracycline and 50.0% to erythromycin (Table 2.5).

Site3. Of the eight *E. faecium* from five sludge samples, 25.0% were resistant to erythromycin, 12.5% to both doxycycline and quinupristin/dalfopristin. Of the eight isolates from five house flies, 25.0% were resistant to tetracycline and 12.5% to ampicillin (Table 2.5).

Site 4. Among the 19 *E. faecium* from nine sludge samples, 21.1% were resistant to erythromycin, 10.5% to tetracycline and 5.3% to both nitrofurantoin and quinupristin/dalfopristin. Of the 10 isolates from six house flies, 30.0% were resistant to tetracycline and 10.0% to both erythromycin and ciprofloxacin (Table 2.5).

2.4.4 Horizontal transfer assays for antibiotic resistance genes

Eight multiple-resistant *E. faecalis* isolates from six Site 1 house flies (three from offsite HF, five from onsite HF) were selected for AR gene horizontal transfer assays using broth and filter mating for gentamicin, streptomycin, tetracycline, doxycycline and erythromycin resistance traits. All of the isolates tested resulted in transconjugants to at least one of the antibiotics at transconjugant/donor (T/D) rates ranging from 2.9×10^{-8} to 7.3×10^{-3} (Tables 2.6, 2.7). Three of the eight isolates transferred all resistances tested in broth and/or filter assays at T/D rates of 6.9×10^{-7} to 7.3×10^{-3}. Among the five isolates resistant to all five antibiotics tested, AR gene transfer occurred among an average of three of the agents during broth mating and four during filter mating. During broth mating, streptomycin resistance was transferred most often (5/8, 62%) at rates from 1.1×10^{-6} to 5.5×10^{-3}. During filter mating assays, all isolates tested (8/8) transferred doxycycline resistance at rates from 8.5×10^{-8} to 7.3×10^{-3} (Tables 2.6, 2.7).

Two isolates of *E. faecium* were tested for transconjugation that were resistant to ampicillin, ciprofloxacin, erythromycin, streptomycin and tetracycline. *E. faecium* ATCC 19454 was used as the recipient. No horizontal transfer of resistance genes was observed.

2.4.5 *E. faecalis* genotypic and phenotypic virulence

Site 1. All *E. faecalis* from Site 1 were tested genotypically with multiplex PCR for *gelE*, *asa1*, *esp* and *cylA*. Virulence phenotypic tests were performed for gelatinase, aggregation substance and cytolysin activity. No phenotypic test was performed for enterococcal surface protein.

Among *E. faecalis*, *gelE* was common from all three sources (sludge 95.5%, onsite HF 93.3% and offsite HF 93.9%) followed by *asa1* (sludge 68.2%, onsite HF 50.0% and offsite HF 36.7%), *cylA* (sludge 19.3%, onsite HF 6.7% and offsite HF 10.2%) and *esp* was the least common (sludge 2.3%, onsite HF 0.8% and offsite HF 15.3%) (Fig. 2.7). When statistically comparing the overall prevalence of the genes among *E. faecalis* from the three sources, *gelE* was not significantly different; *asa1* was not different from sludge and onsite house flies but different ($P < 0.01$) from offsite house flies; *esp* was not different from sludge and onsite house flies but different ($P < 0.05$) from offsite house flies; and *cylA* did not differ among the three sources (Fig. 2.7).

Among the *gelE* isolates, the majority [sludge 74/84 (88.1%), onsite HF 99/112 (88.4%), and offsite HF 75/92 (81.5%)] exhibited the strong gelatinase phenotype (Fig. 2.8). Among *asa1* positive isolates, 7/60 (11.7%) from sludge, 10/60 (16.7%) from onsite HF and 1/36 (2.7%) from offsite HF exhibited the clumping phenotype (Fig. 2.9). Among *cylA* positive isolates, 1/17 (5.9%) from sludge, 1/8 (12.5%) from onsite house flies and 3/10 (30.0%) from offsite house flies exhibited beta hemolysis (Fig. 2.10).

Sites 2, 3, 4. For Sites 2, 3, and 4, only gelatinase phenotypic screening was performed, i.e. no genotypic characterization of any of the genes and no phenotypic tests for aggregation substance, cytolysin or enterococcal surface protein were performed. With the exception of sludge isolates from Site 2, the majority of isolates from both sources (sludge or house flies)

exhibited either the strong or weak gelatinase phenotype (Fig. 2.11). For all locations the strong gelatinase phenotype was most commonly observed over weak gelatinase ranging from 7/31 (22.6%) of sludge isolates at Site 2 to 11/13 (84.6%) of sludge isolates at Site 4 (Fig. 2.11).

2.4.6 *E. faecium* genotypic and phenotypic virulence

Site 1. All *E. faecium* from Site 1 were tested with multiplex PCR for *gelE, esp* and *cylA.* Because *asa1* has not been found in *E. faecium*, no genotypic or phenotypic screening was done for this gene/factor. Of the 48 isolates from the three sources (sludge, onsite HF, offsite HF) 2/32 (0.1%) isolates from offsite HF were positive for *gelE* (data not shown). All other isolates were negative for *gelE, esp* and *cylA* genes (data not shown).

Virulence phenotypic tests were performed for gelatinase and cytolysin activity. Among the 12 *E. faecium* from sludge, none expressed either of the virulence phenotypes. Two of four (50%) isolates from onsite house flies expressed weak genatinase and all were negative for hemolysis (cytolysin) activity. Among offsite house fly *E. faecium*, 8/32 (25%) expressed the weak gelatinase phenotype and all were negative for hemolysis (data not shown).

Sites 2, 3, 4. For Sites 2, 3, and 4, only gelatinase phenotypic screening was performed; none of the isolates from these sights were genotypically screened for virulence genes or for the hemolysis phenotype assay. Weak gelatinase phenotype was exhibited by 13 of 33 (39.4%) sludge isolates and from 9 of 24 (26.5%) house fly isolates (data not shown).

2.4.7 Assessment of clonality of isolates from Site 1

Isolates from Site 1 were genotyped using pulsed-field gel electrophoresis (PFGE) to determine their clonality within and among the three primary sources, sludge, onsite house flies and offsite house flies. From the 2009 season, 40 *E. faecalis* and 13 *E. faecium* were genotyped. Overall diversity was high both among and within the sources (majority between 65-85%

similarity) (Fig. 2.12, 2.13). One clone was recovered from two separate sludge samples collected one week apart (Fig. 2.12). Another two clones from the same sludge sample were greater than 95% similar but no other similar isolates were identified. Further, there was little to no apparent grouping of isolates by source.

Thirteen *E. faecium* from offsite house flies were analyzed (Fig 2.13). Again, there was overall considerable diversity (65-85%) among isolates and no clonal matches from different flies. Two groupings of *E. faecium* consisting of five isolates that were 95-100% similar but came from the same fly (Fig. 2.13).

In 2010, efforts were made to increase the likelihood of identifying similar strains by genotyping isolates collected on the same day. Two sampling dates were selected (21 Jun and 5 Jul) where eight or more isolates were available from each of the three sources. A total of 51 *E. faecalis* were genotyped. A high level of genotypic variation was again observed among the isolates, however, three clonal matches involving eight isolates were identified between bacteria recovered from sludge and onsite house flies (Fig. 2.14).

2.5 DISCUSSION

With the rise of AR bacteria in both clinical and agricultural environments, better understanding of the ecology of the microbes and antibiotic resistance genes is crucial for effective risk assessment, mitigation of pathogen/gene spread and better antimicrobial management. Wastewater treatment facilities play a significant role in the ecology of many microbes (Martins da Costa et al. 2006, Schluter et al. 2007). These operations receive a number of AR bacteria and active proliferation of these bacteria as well as horizontal transfer of AR determinants has been observed (Marcinek et al. 1998, Martins da Costa et al. 2006, Schluter et al. 2007). Due to easy access of house flies to WWTF, the fly may play a substantial role in

dissemination of clinically significant bacteria. The focus of this study was to employ multiple phenotypic and genotypic approaches to characterize enterococci from sewage sludge and house flies assessing the hypothesis that the flies acquire and disseminate clinically important enterococci in this environment.

Enterococci were isolated from 94% of sludge samples from the four sites at a concentration of 10^6 CFU/g. This prevalence and concentration is comparable to other studies that have sampled sewage for the bacteria (Blanch et al. 2003, Ferreria da Silva et al. 2006, Martins da Costa et al. 2006, Ahmed et al. 2008, Nagulapally et al. 2009) and likely provided an abundant source of the bacteria to flies. Sixty six percent of house flies were positive for enterococci at a concentration of 10^3 CFU/fly. This concentration is comparable to previous surveys, though the overall prevalence among flies is lower. In other studies enterococci prevalence in the house flies occurred at rates of 90-98% in environments such as cattle feedlots, swine operations, restaurants and other urban and rural locations (Macovei and Zurek 2006, Chakrabarti et al. 2010, Ahmad et al. 2011). Particularly among animal production sites, the high prevalence in flies may be due largely to abundant animal manure and contamination of multiple surfaces with manure.

During the first season of the study (2008), all enterococcal isolates chosen from selective media plates were identified to species level. Due to the overall prevalence of *E. faecalis* and *E. faecium* (77%) as well as the clinical significance of these two species, they were the exclusive focus during subsequent seasons. Among these two, *E. faecalis* was the most prevalent species both from sludge (78%) and house flies (83%). This prevalence is consistent with previous surveys of sewage. Nagulapally et al. (2009) sampled raw sewage influent from a northeastern Kansas WWTF (Site 3 of this study) and found *E. faecalis* the most abundant species followed

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by *E. casseliflavus* and *E. faecium*.   Also, Blanch et al. (2003) observed *E. faecalis* and *E. faecium* as the most prevalent enterococci among sewage samples throughout Europe.

Of the four WWTF involved in the study, Sites 2, 3 and 4 were most similar in that they received and processed only human sewage. Site 1 was unique in receiving industrial waste from a commercial sausage plant. Sludge sampled from this site consisted entirely of the solids (meat waste) from this source, which were not mixed with residential sewage.   The abundance of fly activity, amount of meat waste and prevalence of antibiotic resistant and virulent enterococci made this location of particular interest. As such, more extensive sampling was conducted at Site 1 and it offered the best opportunity to assess the study hypothesis. The sausage facility did not actively slaughter animals but received meat (beef, pork and poultry) to be used in the final product. Therefore, there were multiple types of meat that likely arrived from multiple sources. Enterococci from the sludge of Site 1 were therefore likely of animal rather than human origin so it is appropriate to compare enterococci findings of this site to those from meat processing facilities and their products. Overall, enterococci are common among many food items including various meats. This has been attributed to the association of enterococci as commensals from the source animals and the hardiness of the bacteria, which allows them to survive the high NaCl, nitrite concentrations and temperatures associated with meat/sausage processing (Giraffa 2002, Martin et al. 2005, 2008).

The proportion of *E. faecalis*/*E. faecium* at this site was 88/12%, which is similar to other studies that have surveyed enterococci at meat processing operations (beef, pork, poultry) and from associated meat products. In most of these studies, *E. faecalis* was dominant followed by *E. faecium* (Knudston and Hartman 1993, Devriese et al. 1995, Quednau et al. 1998, Hayes et al. 2003, Peters et al. 2003, Martin et al. 2008, McGowan-Spicer et al. 2008). In the current study,

the species composition among sludge and house flies were comparable with *E. faecalis* as the most abundant species from both sources as predicted (Fig. 2.2).

The enterococci from Site 1 were more frequently antibiotic resistant than those of Sites 2, 3, and 4 (Fig. 2.3). The predominance of resistance observed among *E. faecalis* was to tetracycline and doxycycline (tetracyclines), streptomycin and gentamicin (aminoglycosides) and erythromycin (macrolide). It has been noted that the enterococcal resistance patterns from food items reflects the use of antimicrobials in the source animal (Lukasova and Sustackova 2003, Silbergeld 2008). Each of the antimicrobial classes that enterococci in this study were commonly resistant to are used for growth promotion in food animals (Silbergeld 2008). Further, tetracycline and erythromycin are commonly administered to animals as well as humans (Silbergeld 2008). Tetracycline resistance, which was the most prominent among enterococci from this site, is also commonly reported among enterococci from various meat products (Hayes et al. 2003, Peters et al. 2003, Ferreira et al. 2006, McGowan et al. 2006, McGowan-Spicer et al. 2008, Ogier and Serror 2008). Erythromycin and gentamicin resistant enterococci are widespread in pork (McGowan et al. 2006), beef and chicken (Koluman et al. 2009) meat products. High level resistance to aminoglycosides (streptomycin, gentamicin) is common among enterococci from food animals and associated meat products (Butaye et al. 2000, Koluman et al. 2009, Hammerum et al. 2010).

There was good overall match among *E. faecalis* antibiotic resistance profiles from sludge and house flies captured onsite with the resistance prevalence from flies consistently below (10-22%) that from sludge (Table 2.3). Further, there was high concurrence between the two sources when considering the specific combinations of resistances (Table 2.4). These observations are consistent with the hypothesis that the flies acquired the bacteria from the

sludge. Further, since the flies likely did not develop in the sludge, they must have arrived at the WWTF from other sites and likely carried other enterococci; explaining why the prevalence was lower in the flies compared to sludge.

Another noteworthy characteristic of *E. faecalis* from Site 1 was the prevalence of virulence factors, particularly for gelatinase (*gelE*) and aggregation substance (*asa1*) (Fig. 2.7-9). This prevalence of putative virulence factors is common among food-animal enterococci. Among 60 strains of *E. faecalis* from Spanish sausage, all were positive for *asa1* and *gelE*, and > 90% for *esp* (Martin et al. 2005).   Among *E. faecalis* from a wide range of food products (fruits, vegetables and various meats) the prevalence of *gelE*, *esp*, *asa1* and *cylA* was 85%, 83% 53% and 11%, respectively (McGowan-Spicer et al. 2008). Additionally, the virulence data for *E. faecalis* are consistent with the hypothesis that the flies obtained *E. faecalis* from sludge. Both genotypic and phenotypic virulence patterns were similar for each source as expected (Fig. 2.7-11).

Genotyping of *E. faecalis* collected in 2009 did not reveal clonal matches between sludge and house flies (Fig. 2.12).   This is likely due to the large genotypic diversity among sludge isolates due to various sources in the meat processing plant. Due to this diversity, genotyping efforts of *E. faecalis* collected during 2010 focused on only two collection dates in an effort to detect clonal matches among the respective sources. Among the 2010 isolates, three clonal matches between sludge and onsite house flies were detected (Fig. 2.14). This is the best evidence to support the study hypothesis that flies acquire enterococci from wastewater treatment facilities. The high level of genotypic diversity among sludge isolates is likely best explained by considering the putative sources of the bacteria. Enterococci at this site likely originated from all meat sources (beef, pork, turkey and chicken), and were likely obtained from multiple suppliers. Another primary contributor to the diversity was the volume of sludge received and stored.   The

facility processed a daily estimated 1.5 cubic meters of sludge and approximately 7.5 cubic meters was stored before weekly removal. This represents an abundant amount of solids available for fly contact. Therefore, given the diversity of the source and volume received and processed the genotypic diversity of the substrate is understandable. Despite this, we found evidence of some transient clonal propagation at the sausage facility. Among the 2009 isolates genotyped, there was one clonal match among two sludge isolates collected a week apart. The samples were not collected from the same storage container, therefore it is expected that there were some persistent strains originating from the sausage plant. Other studies of food processing facilities have noted limited persistence of enterococci. Templer et al. (2007) sampled raw milk cheeses for enterococci from two artisan cheese production plants over a five month period. They observed matching PFGE profiles among *E. faecalis* for periods of 1-2 months and attributed this to a common source at the respective plants.

A noteworthy aspect of *E. faecalis* given the genotypic variation is the similarity of antibiotic resistance and virulence among the isolates. This is likely a result of various sources of the bacterium that are under similar antibiotic pressure, which commonly occurs among the commensals in food production animals (Silbergeld 2008). It has been noted that enterococci with similar resistance profiles in the same environment can be quite genotypcially diverse (Templer et al. 2008). This phenomenon has been attributed to similar selective pressure on enterococci as well as a high degree of horizontal gene transfer. The apparent efficiency of gene exchange has led to the conclusion that the genes themselves are more likely to spread over the bacteria (Butaye et al. 2000, Kotzamanidis et al. 2009, Hammerum et al. 2010).

Eight multiple-resistant *E. faecalis* isolates from flies were selected for *in vitro* antibiotic resistance conjugation assays. Transfer of one or more resistance determinants was observed

among all *E. faecalis* at transconjugant/donor (T/D) rates from 2.9 x 10^{-8} to 7.3 x 10^{-3} (Tables 2.6, 2.7). Enterococci are well known as AR gene reservoirs and readily transfer genes both intra and interspecifically (Kotzamanidis et al. 2009, Hegstad et al. 2010). A number of mobile genetic elements (MGE) such as plasmids and transposons are present in enterococci which facilitate AR gene transfer (Weaver et al. 2002, Top et al. 2008). Further, the house fly digestive tract has been demonstrated to be a conducive environment for conjugal transfer of antibiotic resistance genes among *E. faecalis* (Akhtar et al. 2009). In that study, *E. faecalis* OG1RF with the tetracycline resistance gene *tetM* on the pheromone responsive plasmid pCF10 served as a donor strain and *E. faecalis* OG1SSP (plasmid free) as a recipient. Both strains were fed to house flies and transconjugants of the two strains were observed within 24 h of exposure at T/D rates of 8.6 x 10^{-5} to 4.5 x 10^{1} (Akhtar et al. 2009). Gene transfer as well as clonal propagation in the house fly digestive tract (Chapter 3) represent two ways of amplifying clinically significant enterococci and associated resistance determinants and may lead to enhanced house fly vector competence for these bacteria.

E. faecium were isolated at Site 1 much less frequently than *E. faecalis* and conclusions regarding house fly acquisition and dispersal of this bacterium are less clear. From Site 1, only 12 *E. faecium* from four sludge samples and four from four onsite house flies were isolated (Table 2.1). The greatest number (32) of *E. faecium* associated with Site 1 were obtained from offsite house flies. Among these, moderate levels of resistance were observed to tetracycline and ciprofloxacin (Table 2.5). The disparity of both the prevalence of *E. faecium* and antibiotic resistance patterns between Site 1 sludge and offsite house flies suggests that the *E. faecium* in the flies did not originate from the WWTF.

As outlined above, numerous independent measures of *E. faecalis* from Site 1 support the hypothesis that house flies acquire the bacterium at the facility. The other portion of the hypothesis involves fly dispersal from the wastewater facility and dissemination of AR enterococci. Here the data are less clear but provide circumstantial evidence of house flies carrying enterococci from the WWTF to distances up to 2 km. Though the overall prevalence of antibiotic resistance among *E. faecalis* from offsite house flies was significantly lower, the profile of specific antibiotics that the bacteria were resistant to matched that of *E. faecalis* from both sludge and onsite house flies. The same general trend was found among virulence genes and phenotypes. There were no clonal matches observed by PFGE among offsite flies to either sludge or onsite flies. This is not unexpected given the level of diversity of *E. faecalis* from sludge. Further, flies sampled away from the WWTF could have migrated from areas other than the WWTF and so a level of dilution is expected. Finally, it is possible that enterococci acquired from the WWTF might have diminished in prevalence in the time it took the flies to migrate to these offsite locations and could contribute to the lower resistance prevalence.

Sites 2, 3, and 4 received exclusively human sewage and therefore represent more typical wastewater operations. Among the three sites, *E. faecalis* antibiotic resistance occurred to the same five antibiotics as observed at Site 1, tetracycline, doxycycline, erythromycin, gentamicin and streptomycin, though at a lower overall prevalence (Fig. 2.3). This pattern of phenotypic resistance is comparable to other studies assessing *E. faecalis* resistance from sewage (Blanch et al. 2003, Ferreira da Silva et al. 2006, Martins da Costa et al. 2006). Nagulapally et al. (2009) screened enterococci from raw influent (study site was Site 3 from this present study) for resistance to ciprofloxacin and vancomycin. No resistance to ciprofloxacin was observed among enterococci from samples collected in the summer as no resistance was observed from sludge at

this location in the present study. Nagulapally et al. did isolate vancomycin resistant enterococci (VRE) (32 mg/L vancomycin) from 2% of the enterococcal population sampled from raw influent. No VRE were isolated in the present study, perhaps due to less extensive sampling of the sludge. Blanch et al. (2003) observed erythromycin resistance from raw sewage among *E. faecalis* of 52 and 76% from samples from Sweden and Spain, respectively. Erythromycin resistance among *E. faecalis* in this present study varied from 14% from sludge at Site 3 to 69% from Site 4 sludge. Ferreira da Silva et al. (2006) sampled raw wastewater from a Portugal WWTF and observed resistance prevalence among *E. faecalis* of 33%, 40% and 57% for ciprofloxacin, erythromycin and tetracycline, respectively. These values match well with the present study for erythromycin and tetracycline, but were lower for ciprofloxacin resistance. A survey of enterococci (species not identified) from influent, treated effluent and sludge at 14 WWTF throughout Portugal revealed resistance to ampicillin (4%), vancomycin (0.7%), tetracycline (37%), erythromycin (25%), gentamicin (3%), nitrofurantoin (23%) and ciprofloxacin (15%) (Martins da Costa et al. 2006). These resistance rates compare well with those observed in this study for *E. faecalis* and *E. faecium*, with the exception of the greater percentage of ciprofloxacin resistance (15%) observed by Martins da Costa et al. No resistance to streptomycin was observed from *E. faecalis* at Sites 2 and 3 (Table 2.3). This was the case among isolates from both sludge and house flies as would be expected if the sludge is a source of enterococci for flies. Further, Sites 2 and 3 had similar resistance profiles to tetracycline, doxycycline, gentamicin and erythromycin among the two sources (Table 2.3).   Resistance patterns from sludge and house flies were less similar among *E. feacalis* at Site 4 (Table 2.3). This may suggest an effect based on the site where the ability of flies to acquire enterococci varies among WWTF. This is likely given the differences in structure, operation and

handling/storage of waste among the four sites surveyed. However, it should also be noted that the fewest *E. faecalis* (13) of all four sites were isolated from Site 4 sludge. At this site, more *E. faecium* than *E. faecalis* were recovered from sludge (Table 2.1). Therefore, conclusions based on the *E. faecalis* population at this site suffer from a deficiency of characterized isolates. Moreover, the number of isolates from Sites 2, 3 and 4 was lower compared to that of Site 1 and therefore it is difficult to make statements about enterococcal diversity. Gelatinase phenotypes of *E. faecalis* at these sites were categorized as negative, weak and strong (Macovei et al. 2009). Prevalence from sludge and house flies for each of the sites varied in similarity among the three sites. Since there were no other genotypic or phenotypic analyses of virulence factors done on these isolates there are fewer data available to compare the sources.

Similar to Site 1, fewer *E. faecium* than *E. faecalis* were recovered from Sites 2, 3 and 4 making conclusions regarding ecology of these bacteria difficult. Of the three sites, the greatest number of *E. faecium* (19) were isolated from Site 4 sludge (Table 2.1). *E. faecium* from sludge and house flies at this site were resistant to tetracycline and erythromycin but differed in resistance to quinupristin/dalfopristin and ciprofloxacin (Table 2.5).

The significance of house flies in the ecology of various microbes has become clearer over the past decade. Recent studies that have specifically considered the interaction of flies and antibiotic resistant enterococci have provided strong evidence that house flies regularly acquire the bacteria in environments such as poultry and swine operations (Graham et al. 2009, Ahmad et al. 2011). Further, due to the high mobility of house flies, AR enterococci from these environments may be dissemination to rural and urban areas, which could facilitate clonal spread and dispersal of associated AR genes. This study assessed another environment where house flies may play a role in AR enterococcal ecology. Observations from various independent

measures support the hypothesis that flies acquire and disseminate AR enterococci from wastewater treatment facilities. The best evidence originates from Site 1, which apparently involved primarily a food animal source of enterococci. Though the outset goal of the study was to investigate a human source of AR enterococci to flies, the nature of waste processed at Site 1 points to yet another animal source of the AR bacteria. Despite this, the results are broadly applicable to more common wastewater treatment facilities that receive human sewage. Moreover, evidence to support the hypothesis was obtained at Sites 2, 3, and 4 as well, which did exclusively receive human sewage.   The overall prevalence of virulence and antibiotic resistance among enterococci was lower among the latter three sites. However, this may be different for facilities that receive human sewage from major urban communities with large hospitals. At these sites, a higher rate of AR enterococci as well as an elevated level of antimicrobials, which could induce AR gene spread, would be expected among the sewage influent. Future studies in this and other environments would benefit from a more extensive look at the extent and prevalence of fly dispersal as well efforts to quantify any enterococcal contamination that could result in human exposure to the bacteria.

It should be noted that while this study focused on enterococci, there are a number of other bacteria of medical/veterinary interest and could be acquired and disseminated by house flies from WWTF as well. Examples of bacteria that have been cultured from wastewater and at various points along the waste treatment stream include *Escherichia coli* (Sturtevant and Feary 1969, Grabow and Prozesky 1973, Walter and Vennes 1985) *Salmonella typhi* (Grabow and Prozesky 1973, Schluter et al. 2007), *Acintenobacter* spp. (Guardabassi et al. 1998), *Staphylocuccus aureus*, *Legionella pneumophila*, and *Clostridium difficile* (Viau and Peccia 2009). House flies likely play a varying role in the ecology of these bacteria at WWTF as well.

Results from this study serve to enhance our understanding of risks associated with dissemination of AR bacteria. Factors such as the access of house flies to various wastewater treatment processes should be considered when operating and designing new facilities. Further, WWTF management may consider fly control during the peak season of fly activity to limit AR microbe spread.

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2.7 FIGURES AND TABLES

Figure 2.1 Site 1 wastewater treatment facility and nearby locations sampled for house flies

Figure 2.2 Diversity of *Enterococcus* species at four wastewater treatment facilities (all enterococcal isolates identified to species during 2008). A - isolates from Site 1, B - combined isolates from Sites 2, 3, 4.
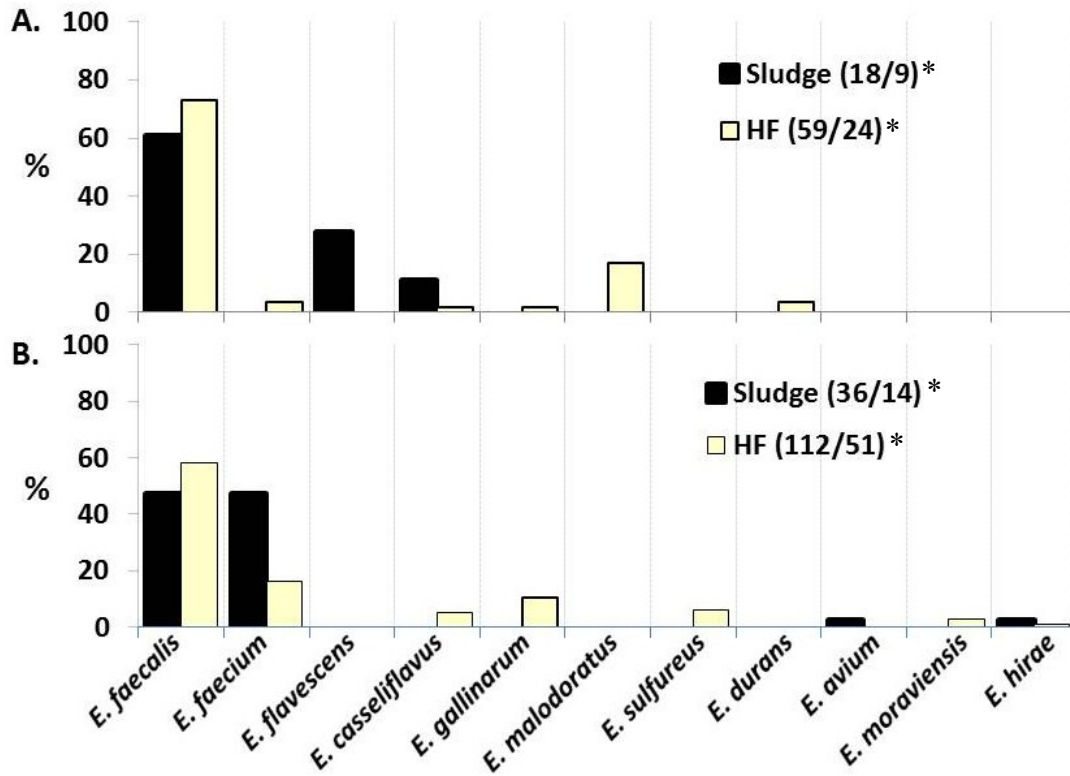
Table 2.1 Prevalence and identification of enterococci isolated from sludge at four wastewater treatment facilities.

Wastewater plant	No. of samples analyzed/ no. positive (%)	CFU/g (mean ± SEM)	Total no. of isolates characterized	No. (%) of isolates	
				E. faecalis	*E. faecium*
Site 1	41/39 (95.1)	$4.9 \pm 1.5 \times 10^6$	100	88 (88.0)	12 (12.0)
Site 2	15/13 (86.7)	$2.6 \pm 1.7 \times 10^5$	37	31 (83.8)	6 (16.2)
Site 3	14/14 (100)	$5.5 \pm 2.6 \times 10^4$	37	29 (78.3)	8 (21.6)
Site 4	19/18 (94.7)	$2.5 \pm 0.6 \times 10^4$	32	13 (40.6)	19 (59.4)
Total or mean	89/84 (94.4)	$2.3 \pm 0.8 \times 10^6$	206	161 (78.2)	45 (21.8)

Table 2.2 Prevalence and identification of enterococci isolated from house flies (HF) at and near four wastewater treatment facilities.

Wastewater plant	No. of HF analyzed/ no. positive (%)	CFU/HF (mean ± SEM)	Total no. of isolates characterized	No. (%) of isolates	
				E. faecalis	*E. faecium*
Site 1	83/56 (67.5)	$4.5 \pm 1.0 \times 10^3$	124	120 (96.8)	4 (3.2)
Near Site 1 (0.7-2 km)	84/51 (60.7)	$1.5 \pm 1.0 \times 10^4$	130	98 (75.4)	32 (24.6)
Site 2	28/16 (57.1)	$3.7 \pm 1.8 \times 10^3$	25	19 (76.0)	6 (24.0)
Site 3	43/30 (69.8)	$1.3 \pm 1.1 \times 10^4$	34	26 (76.4)	8 (23.5)
Site 4	38/28 (73.7)	$7.0 \pm 2.0 \times 10^3$	43	33 (76.8)	10 (23.2)
Total or mean	276/181 (65.6)	$8.9 \pm 3.4 \times 10^3$	356	296 (83.1)	60 (16.9)

Table 2.3 Antibiotic resistance profile of *E. faecalis* from sludge and house flies (HF) at four wastewater treatment facilities. TET-tetracycline, D-doxycyline, S-streptomycin, GM-gentamicin, ERY-erythromycin, CIP-ciprofloxacin, NIT-nitrofurantoin. No resistance was observed to four other antibiotics, ampicillin, vancomycin, linezolid and tigecycline.

WWTF	Source	no. isolates	% resistant						
			TET	D	S	GM	ERY	CIP	NIT
Site 1	Sludge	88	85.2	76.1	35.2	52.3	52.3	0	0
	HF (onsite)	120	71.7	54.2	25.0	40.8	39.2	0	1.7
	HF (offsite)	98	25.5	16.3	6.1	8.2	8.2	0	0
Site 2	Sludge	31	35.5	3.2	0	3.2	22.6	0	0
	HF	19	47.4	15.8	0	5.3	26.3	0	0
Site 3	Sludge	29	31.0	6.9	0	0	13.8	0	0
	HF	26	50.0	30.8	0	0	23.1	0	0
Site 4	Sludge	13	76.9	61.5	61.5	7.7	69.2	7.7	0
	HF	33	6.1	9.1	3.0	3.0	0	0	0

Figure 2.3 Resistance profile of *E. faecalis* to 11 antibiotics from sludge and house flies (HF) at four wastewater treatment facilities. Site 1 resistance profile is contrasted with the combined profiles of Sites 2, 3, and 4. A - isolates from Site 1, B - combined isolates from Sites 2, 3, 4. TET-tetracycline, D-doxycyline, S-streptomycin, GM-gentamicin, ERY-erythromycin, AMP-ampicillin, CIP-ciprofloxacin, VAN-vancomycin, NIT-nitrofurantoin, LZO-linezolid, TGC-tigecycline. Specific profiles for Sites 2, 3, 4 are provided in Table 2.3.

*number of *E. faecalis* isolates/number of samples

96
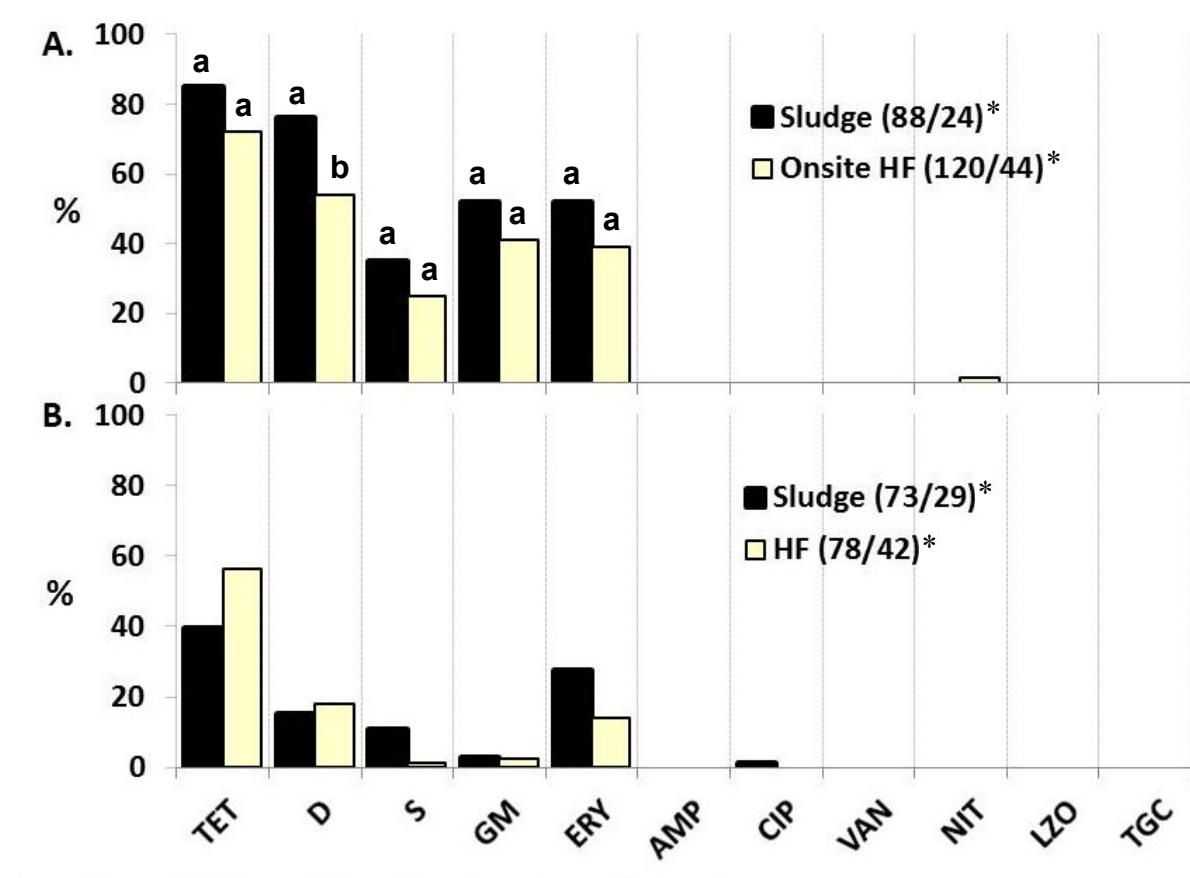
Figure 2.4 Resistance profile of *E. faecalis* to 11 antibiotics from sludge and house flies (HF) at and near Site 1 wastewater treatment facility. TET-tetracycline, D-doxycyline, S-streptomycin, GM-gentamicin, ERY-erythromycin, AMP-ampicillin, CIP-ciprofloxacin, VAN-vancomycin, NIT-nitrofurantoin, LZO-linezolid, TGC-tigecycline.
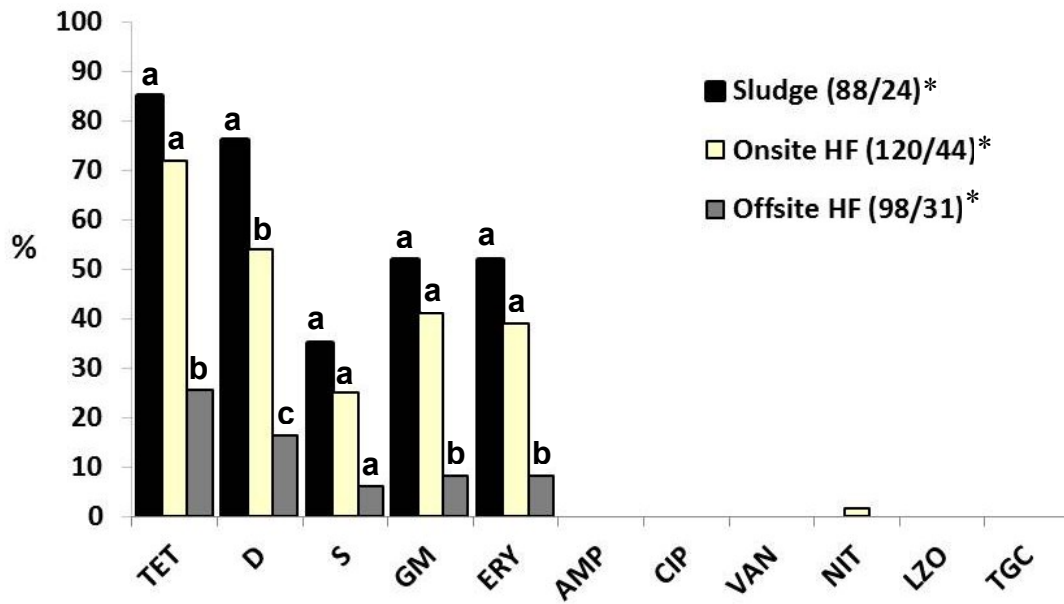
*number of *E. faecalis* isolates/number of samples

Table 2.4 Antibiotic resistance profile among *E. faecalis* from sludge and house flies (HF) onsite and nearby (offsite) of Site 1 wastewater treatment facility. TET-tetracycline, D-doxycyline, ERY-erythromycin, S-streptomycin, GM-gentamicin, NIT- nitrofuratoin.

Resistance profile	Sludge (n=88/24) [a]	HF onsite (n=120/44) [a]	HF offsite (n=98/31) [a]
	no. of resistant isolates (%)	no. of resistant isolates (%)	no. of resistant isolates (%)
TET	6 (6.8)	11 (9.2)	7 (7.1)
ERY	3 (3.4)	2 (1.6)	
GM			5 (5.1)
D	1 (1.1)		
TET, D	11 (12.5)	11 (9.2)	6 (6.1)
TET, S	2 (2.3)	2 (1.6)	
TET, ERY		5 (4.2)	
TET, D, GM	14 (15.9)	15 (12.5)	1 (1.0)
TET, D, ERY	6 (6.8)	6 (5.0)	4 (4.1)
TET, ERY, S	3 (3.4)		2 (2.0)
TET, ERY, NIT		1 (0.8)	
TET, D, S	1 (1.1)		1 (1.0)
D, ERY, GM	1 (1.1)		
TET, D, ERY, GM	8 (9.1)	6 (5.0)	
TET, D, ERY, S	2 (2.3)	1 (0.8)	
TET, ERY, S, GM		2 (1.6)	
TET, D, S, GM		1 (0.8)	
TET, D, GM, NIT		1 (0.8)	
TET, D, ERY, S, GM	22 (25.0)	24 (20.0)	1 (1.0)
Pan-susceptible	8 (9.1)	32 (26.7)	71 (72.5)

[a] number of *E. faecalis*/number of samples

Table 2.5 Resistance profile of *E. faecium* to six antibiotic from sludge and house flies (HF) at four wastewater treatment facilities. TET-tetracycline, D-doxycyline, ERY-erythromycin, AMP-ampicillin, Q/D-quinupristin/dalfopristin, CIP-ciprofloxacin. No resistance was observed to five other antibiotics, streptomycin, gentamicin, vancomycin, linezolid and tigecycline.

| WWTF | source | no. isoloates | % resistant | | | | | | |
			TET	D	ERY	AMP	Q/D	CIP	NIT
Site 1	Sludge	12	0	0	50.0	0	0	0	0
	HF (onsite)	4	25.0	0	0	0	0	25.0	0
	HF (offsite)	32	40.1	3.1	6.3	6.3	0	28.1	3.1
Site 2	Sludge	6	16.7	16.7	16.7	0	0	0	0
	HF	6	66.7	0	50.0	0	0	0	0
Site 3	Sludge	8	0	12.5	25.0	0	12.5	0	0
	HF	8	25.0	0	0	12.5	0	0	0
Site 4	Sludge	19	10.5	0	21.1	0	5.3	0	5.3
	HF	10	30.0	0	10.0	0	0	10.0	0

Figure 2.5 Resistance profile of *E. faecium* to 12 antibiotics from sludge and house flies (HF) at four wastewater treatment facilities. A - Site 1, B - Sites 2, 3, 4 combined. TET-tetracycline, D-doxycyline, S-streptomycin, GM-gentamicin, ERY-erythromycin, AMP-ampicillin, Q/D – quinupristin/dalfopristin, CIP-ciprofloxacin, VAN-vancomycin, NIT-nitrofurantoin, LZO-linezolid, TGC-tigecycline. Specific profiles for Sites 2, 3, 4 are provided in Table 2.5.
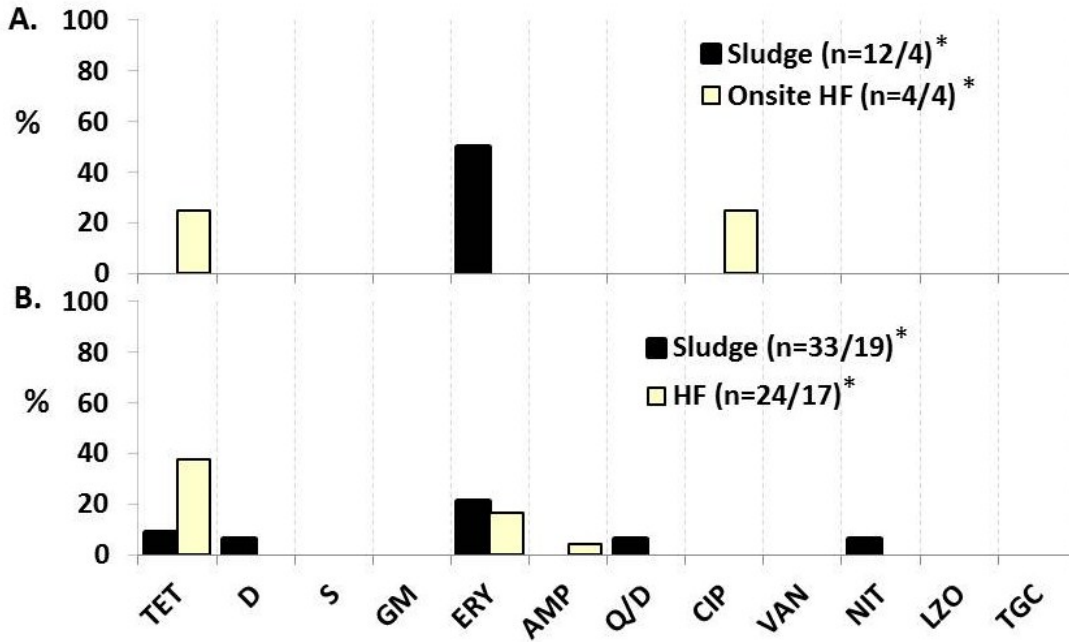
[*] number of *E. faecium*/number of samples

Figure 2.6 Resistance profile of *E. faecium* to 12 antibiotics from sludge and house flies (HF) at and nearby Site 1 wastewater treatment facility. TET-tetracycline, D-doxycyline, S-streptomycin, GM-gentamicin, ERY-erythromycin, AMP-ampicillin, Q/D – quinupristin/dalfopristin, CIP-ciprofloxacin, VAN-vancomycin, NIT-nitrofurantoin, LZO-linezolid, TGC-tigecycline.
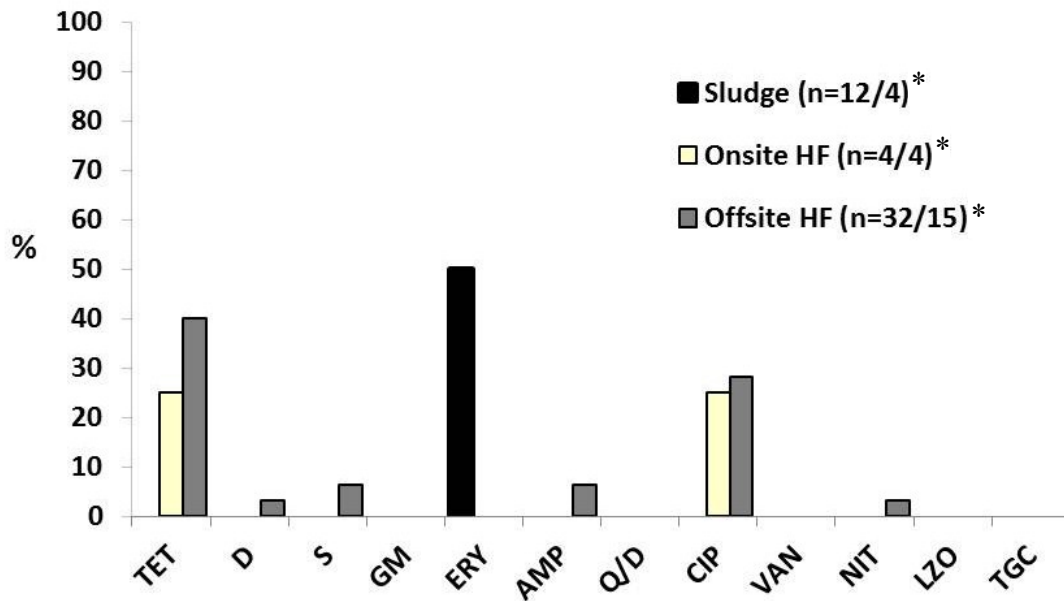
*number of *E. faecium*/number of samples

Table 2.6 Horizontal transfer of antibiotic resistance genes by broth mating among *E. faecalis* from house flies at and near Site 1 wastewater treatment facility. Recipient for streptomycin *E. faecalis* 41-31 (wild isolate), recipient for all other resistance genes *E. faecalis* OG1SSP. OFHF, offsite house fly; ONHF, onsite house fly.

Donor	Transfer rate (T/D)*				
	streptomycin	gentamicin	tetracycline	doxycycline	erythromycin
OFHF 7-2	0	NR^a	0	0	NR^a
OFHF 7-3	1.7×10^{-3}	NR^a	0	0	0
OFHF 7-4	5.5×10^{-3}	NR^a	0	3.6×10^{-7}	2.9×10^{-8}
ONHF 5-4	1.1×10^{-6}	0	6.3×10^{-8}	0	0
ONHF 6-1	0	0	0	0	0
ONHF 8-3	1.9×10^{-6}	2.9×10^{-5}	6.8×10^{-6}	1.3×10^{-6}	1.8×10^{-6}
ONHF 10-1	0	2.9×10^{-5}	3.4×10^{-4}	3.4×10^{-4}	1.4×10^{-4}
ONHF 16-4	1.5×10^{-4}	7.1×10^{-5}	8.2×10^{-6}	8.2×10^{-6}	0

* T, transconjugant; D, donor
^{a} NR, not resistant

Table 2.7 Horizontal transfer of antibiotic resistance genes by filter mating among *E. faecalis* from house flies at and near Site 1 wastewater treatment facility. Recipient for streptomycin *E. faecalis* 41-31 (wild isolate), recipient for all other resistance genes *E. faecalis* OG1SSP. OFHF, offsite house fly; ONHF, onsite house fly.

Donor	Transfer rate (T/D)*				
	streptomycin	gentamicin	tetracycline	doxycycline	erythromycin
OFHF 7-2	0	NR[a]	9.3×10^{-8}	5.3×10^{-7}	NR[a]
OFHF 7-3	0	NR[a]	0	1.8×10^{-7}	3.9×10^{-7}
OFHF 7-4	0	NR[a]	0	3.5×10^{-6}	1.1×10^{-7}
ONHF 5-4	1.4×10^{-7}	2.7×10^{-7}	1.1×10^{-7}	1.2×10^{-7}	0
ONHF 6-1	0	3.1×10^{-7}	1.4×10^{-7}	8.5×10^{-8}	0
ONHF 8-3	0	3.3×10^{-3}	9.1×10^{-4}	4.9×10^{-3}	2.2×10^{-3}
ONHF 10-1	1.1×10^{-6}	2.1×10^{-3}	3.1×10^{-4}	7.3×10^{-3}	1.2×10^{-3}
ONHF 16-4	0	1.3×10^{-4}	4.6×10^{-6}	1.8×10^{-5}	6.9×10^{-7}

* T, transconjugant; D, donor
[a] NR, not resistant

Figure 2.7 Virulence genotypic profile of *E. faecalis* from Site 1 wastewater treatment facility. HF - house flies, *gelE* – gelatinase, *asa1* – aggregation substance, *esp* – enterococcal surface protein, *cylA* – cytolysin.
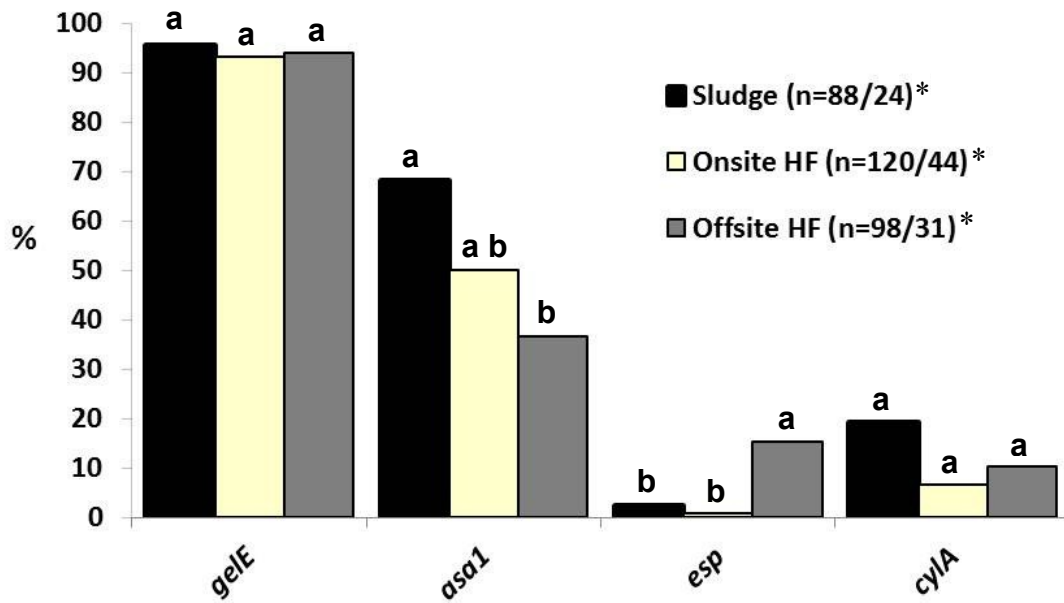
* number of *E. faecalis* isolates/number of samples

Figure 2.8 Prevalence of *gelE* among *E. faecalis* and correlation of gelatinase phenotype from Site 1 wastewater treatment facility. Phenotypic profile based on percent of isolates positive for *gelE*. HF - house flies, *gel*E – gelatinase gene present, SG - strong gelatinase phenotype, WG - weak gelatinase phenotype, NG - negative gelatinase phenotype.
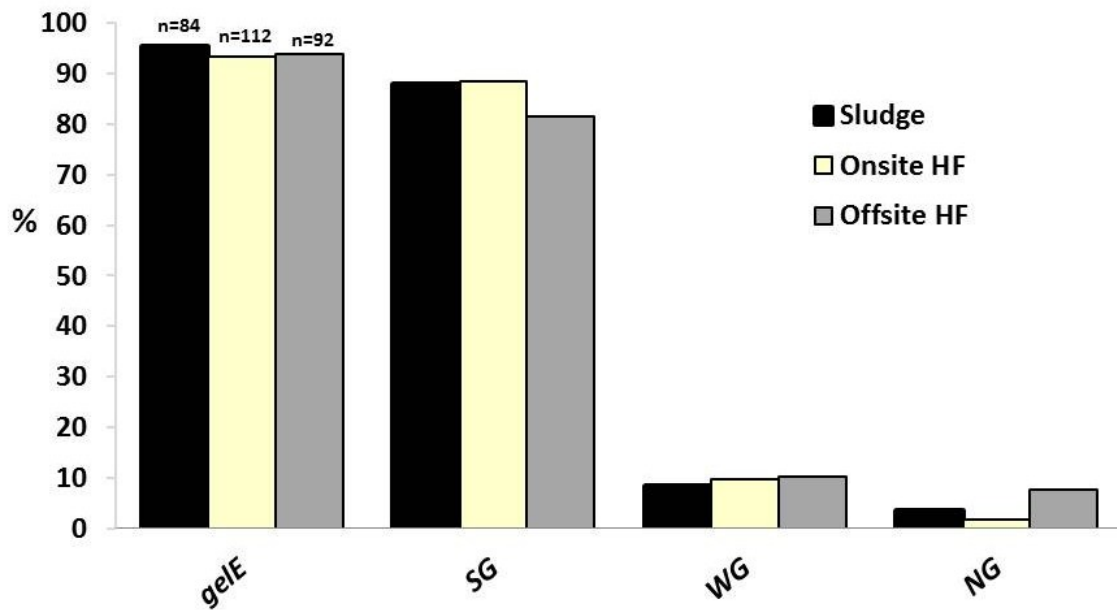
Figure 2.9 Prevalence of *asa1* among *E. faecalis* and correlation of aggregation phenotype from Site 1 wastewater treatment facility. Phenotypic profile based on percent of isolates positive for *asa1*. HF - house flies, *asa1* – aggregation substance gene present, PC – clumping phenotype, NC – negative clumping phenotype.
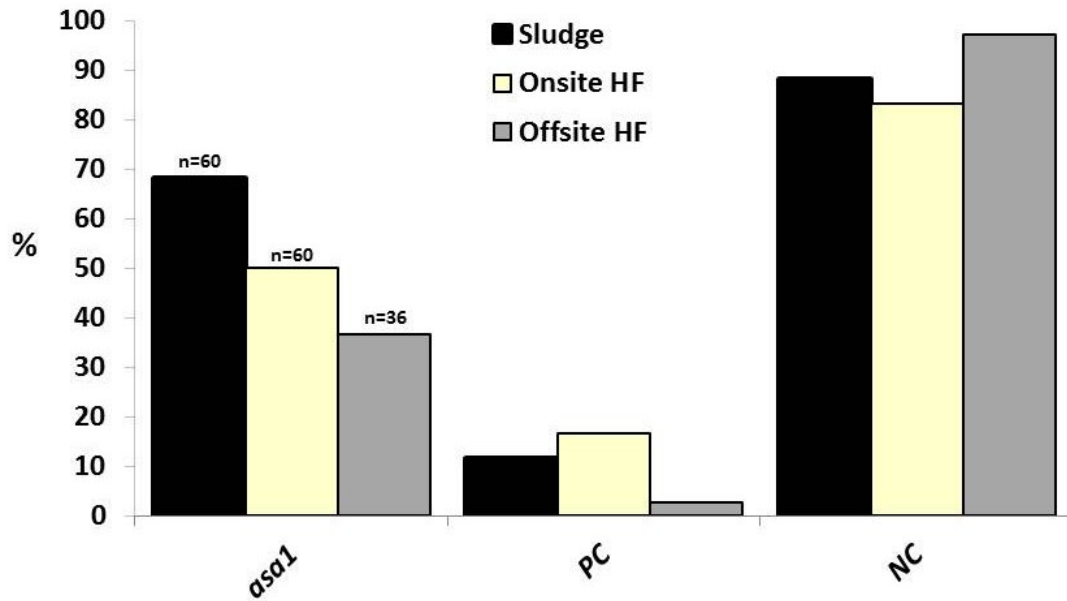
Figure 2.10 Prevalence of *cylA* among *E. faecalis* and correlation of hemolysis phenotype from Site 1 wastewater treatment facility. Phenotypic profile based on percent of isolates positive for *cylA*. HF - house flies, *cylA* – cytolysin gene present, *β* – beta hemolysis, neg. – negative phenotype.
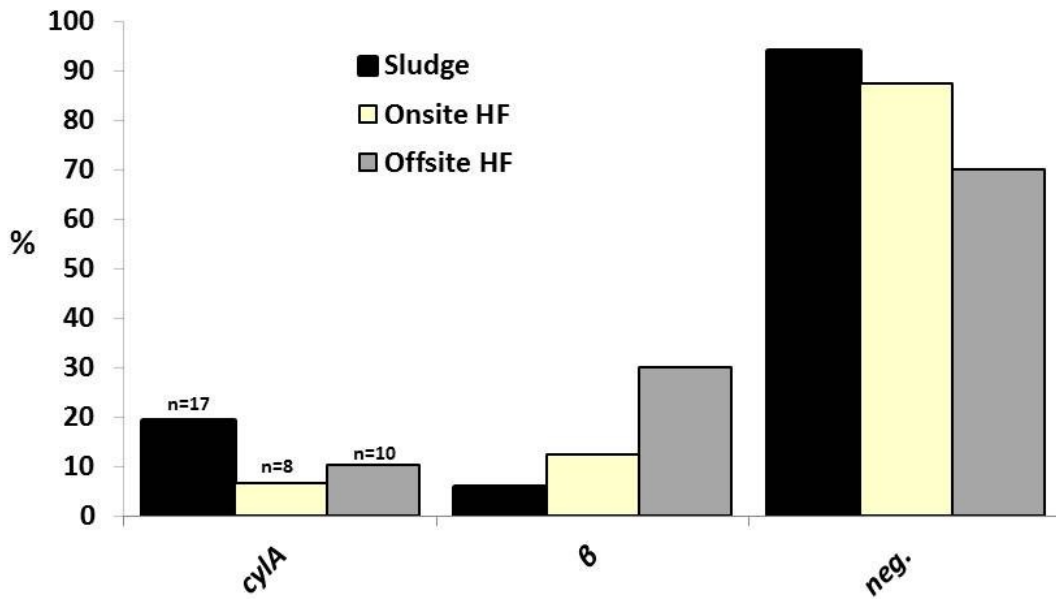
Figure 2.11 Gelatinase phenotypic profiles of *E. faecalis* from Sites 2, 3, 4 wastewater treatment facilities. A = Site 2, B = Site 3, C = Site 4. HF - house flies, SG - strong gelatinase phenotype, WG - weak gelatinase phenotype.
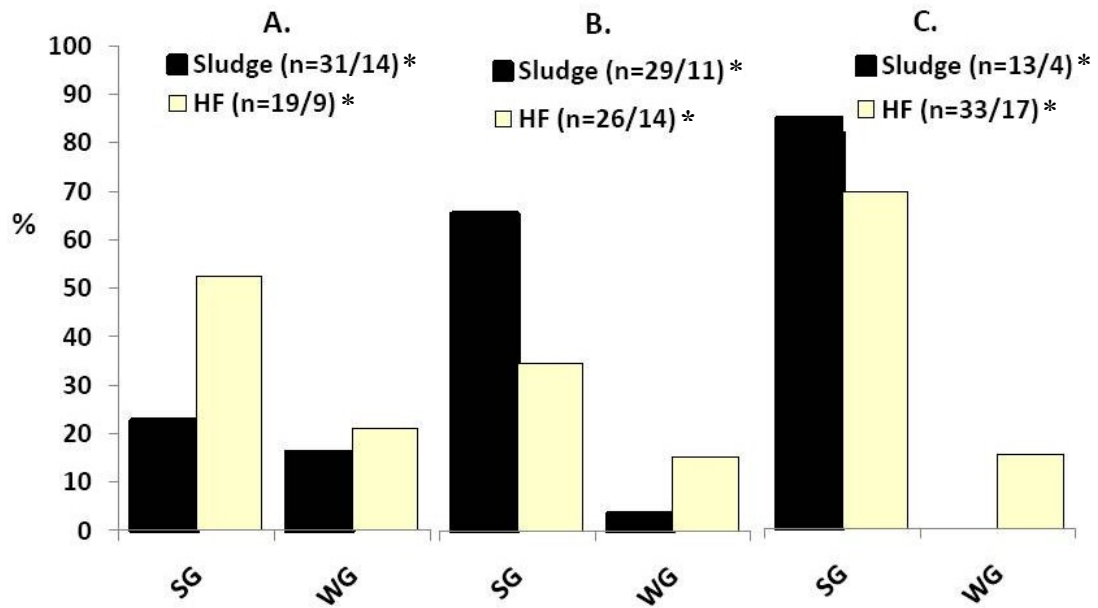
* number of *E. faecalis* isolates/number of samples

Figure 2.12 Pulsed-field gel electrophoresis (PFGE) dendogram of *E. faecalis* based on *Apa1* restriction from sludge, house flies (HF) onsite and HF offsite (restaurant and apartments) of Site 1 wastewater treatment facility collected during 2009.

Isolate	Source	Date collected
SLE 2-6	Sludge	01 Jul
SLE 3-3	Sludge	01 Jul
SLE 4-3	Sludge	07 Jul
HF 2-3	HF - onsite	24 Jun
SLE 1-2	Sludge	24 Jun
HF 16-5	HF - onsite	14 Jul
HF 6-1	HF - onsite	01 Jul
SLE 3-2	Sludge	01 Jul
SLE 4-6	Sludge	07 Jul
SLE 2-3	Sludge	01 Jul
RI 13-1	HF - offsite, restaraunt	20 Jul
HF 10-7	HF - onsite	07 Jul
HF 6-4	HF - onsite	01 Jul
HF 5-8	HF - onsite	01 Jul
RO 6-3	HF - offsite, restaraunt	14 Jul
SLE 5-2	Sludge	07 Jul
HF 10-2	HF - onsite	07 Jul
SLE 1-4	Sludge	24 Jun
HF 10-3	HF - onsite	07 Jul
APT 7-1	HF - offsite, apartment	20 Jul
RO 9-7	HF - offsite, restaraunt	15 Jul
SLE 6-5	Sludge	20 Jul
SLE 10-5	Sludge	11 Aug
SLE 10-7	Sludge	11 Aug
RI 6-4	HF - offsite, restaraunt	14 Jul
HF 17-4	HF - onsite	14 Jul
HF 17-5	HF - onsite	14 Jul
HF 6-2	HF - onsite	01 Jul
SLE 5-5	Sludge	07 Jul
HF 11-4	HF - onsite	07 Jul
RI 8-7	HF - offsite, restaraunt	15 Jul
HF 5-2	HF - onsite	01 Jul
RO 3-4	HF - offsite, restaraunt	08 Jul
APT 7-2	HF - offsite, apartment	20 Jul
SLE 6-2	Sludge	20 Jul
APT 9-4	HF - offsite, apartment	20 Jul
HF 16-3	HF - onsite	14 Jul
RI 14-4	HF - offsite, restaraunt	21 Jul
SLE 9-3	Sludge	11 Aug
SLE 9-7	Sludge	11 Aug

Figure 2.13 Pulsed-field gel electrophoresis (PFGE) dendogram of *E. faecium* based on *Apa1* restriction from house flies (HF) offsite (restaurant and apartments) of Site 1 wastewater treatment facility collected during 2009.

Isolate	Source	Date collected
APT fm 9-6	HF - offsite, apartment	20 Jul 09
APT fm 9-8	HF - offsite, apartment	20 Jul 09
RI Fm 3-5	HF - offsite, restaraunt	14 Jul 09
APT fm 7-6	HF - offsite, apartment	20 Jul 09
APT fm 7-7	HF - offsite, apartment	20 Jul 09
APT fm 7-5	HF - offsite, apartment	20 Jul 09
RO fm 12-1	HF - offsite, restaraunt	20 Jul 09
RO fm 6-7	HF - offsite, restaraunt	14 Jul 09
RO fm 11-4	HF - offsite, restaraunt	20 Jul 09
RO fm 3-5	HF - offsite, restaraunt	08 Jul 09
RI fm 2-2	HF - offsite, restaraunt	08 Jul 09
RO fm 4-11	HF - offsite, restaraunt	14 Jul 09
RI fm 15-3	HF - offsite, restaraunt	20 Jul 09

Figure 2.14 Pulsed-field gel electrophoresis (PFGE) dendogram of *E. faecalis* based on *Apa1* restriction of *E. faecalis* from sludge, house flies (HF) onsite and house flies offsite (RV park and apartments) of Site 1 wastewater treatment facility collected during 2010. Brackets denote clonal matches between isolates from sludge and onsite house flies.

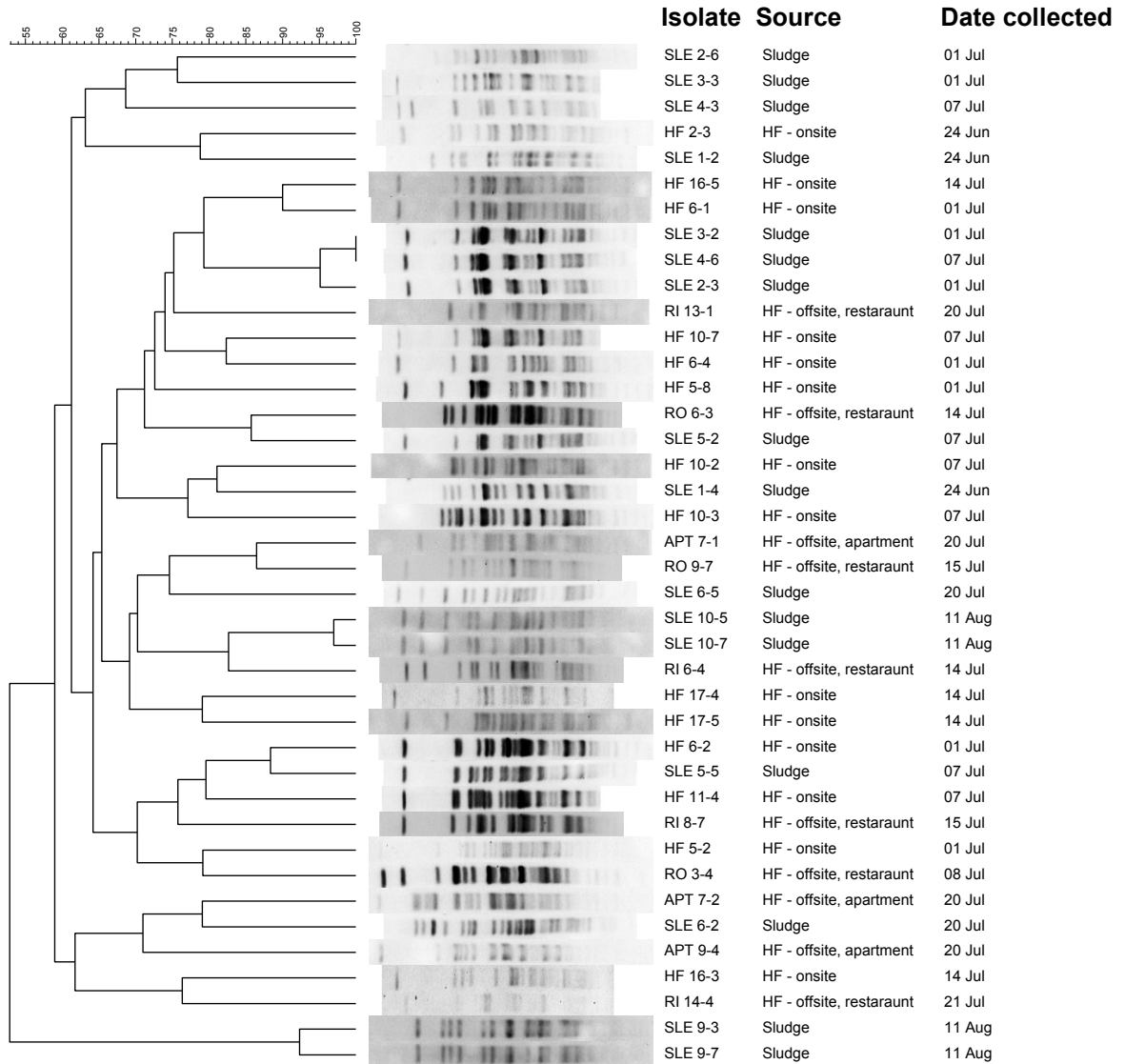| Isolate	Source	Date collected
SLE 10-10-2	Sludge	21 Jun 10
SLE 10-13-1	Sludge	5 Jul 10
HF 10-18-5	HF - onsite	5 Jul 10
HF 10-25-1	HF - onsite	5 Jul 10
SLE 10-13-3	Sludge	5 Jul 10
SLE 10-15-3	Sludge	5 Jul 10
HF 10-15-5	HF - onsite	21 Jun 10
ON 10-11-3	HF - offsite, RV park	5 Jul 10
ON 10-11-1	HF - offsite, RV park	5 Jul 10
HF 10-10-5	HF - onsite	21 Jun 10
SLE 10-5-9	Sludge	21 Jun 10
HF 10-21-5	HF - onsite	5 Jul 10
ON 10-13-1	HF - offsite, RV park	5 Jul 10
APT 10-10-1	HF - offsite, apartment	21 Jun 10
APT 10-13-2	HF - offsite, apartment	21 Jun 10
APT 10-11-2	HF - offsite, apartment	21 Jun 10
APT 10-11-3	HF - offsite, apartment	21 Jun 10
HF 10-18-1	HF - onsite	5 Jul 10
SLE 10-14-1	Sludge	5 Jul 10
SLE 10-14-2	Sludge	5 Jul 10
HF 10-22-5	HF - onsite	5 Jul 10
SLE 10-16-2	Sludge	5 Jul 10
APT 10-10-4	HF - offsite, apartment	21 Jun 10
APT 10-13-4	HF - offsite, apartment	21 Jun 10
SLE 10-11-1	Sludge	5 Jul 10
APT 10-13-3	HF - offsite, apartment	21 Jun 10
SLE 10-4-10	Sludge	21 Jun 10
SLE 10-6-4	Sludge	21 Jun 10
HF 10-10-4	HF - onsite	21 Jun 10
HF 10-15-6	HF - onsite	21 Jun 10
SLE 10-6-5	Sludge	21 Jun 10
APT 10-11-1	HF - offsite, apartment	21 Jun 10
HF 10-10-3	HF - onsite	21 Jun 10
SLE 10-6-1	Sludge	21 Jun 10
SLE 10-7-3	Sludge	21 Jun 10
APT 10-9-1	HF - offsite, apartment	21 Jun 10
SLE 10-4-9	Sludge	21 Jun 10
SLE 10-8-11	Sludge	21 Jun 10
HF 10-28-1	HF - onsite	5 Jul 10
HF 10-23-4	HF - onsite	5 Jul 10
HF10-29-1	HF - onsite	5 Jul 10
HF 10-20-4	HF - onsite	5 Jul 10
SLE 10-15-6	Sludge	5 Jul 10
HF 10-15-4	HF - onsite	21 Jun 10
HF 10-15-8	HF - onsite	21 Jun 10
SLE 10-8-1	Sludge	21 Jun 10
ON 10-11-2	HF - offsite, RV park	5 Jul 10
SLE 10-15-2	Sludge	5 Jul 10
APT 10-14-2	HF - offsite, apartment	5 Jul 10
SLE 10-7-2	Sludge	21 Jun 10
SLE 10-16-4	Sludge	5 Jul 10

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CHAPTER 3: SURVIVAL AND PROLIFERATION OF *ENTEROCOCCUS FAECALIS* IN THE HOUSE FLY DIGESTIVE TRACT

3.1 ABSTRACT

Enterococcus faecalis is an important nosocomial pathogen and house flies have been implicated in the dissemination and transfer of this bacterium in the agricultural as well as urban environment. In this study, the GFP-expressing strain of *Enterococcus faecalis* OG1RF:pMV158 was used to track the fate of the bacterium in the digestive tract of the common house fly, *Musca domestica* (L.), to assess the vector potential of this insect for *E. faecalis* under laboratory conditions. Colony forming unit (CFU) counts were obtained from viable fluorescing *E. faecalis* recovered from mouthparts and digestive tract regions (labellum, foregut, midgut, hindgut) at 1, 4, 8, 24, 48, 72 and 96 h after the initial bacterial exposure. Counts were highest in the midgut at 1 h and declined during the first 24 h. In the labellum, foregut and hindgut, *E. faecalis* concentrations were more variable but overall higher after 24 h. Observations of the digestive tract under a dissecting microscope with UV light revealed that *E. faecalis* peaked in the crop after 48 h. Our data suggest that *E. faecalis* was digested in the midgut; however, microscopy and CFU counts indicated the proliferation in the crop. Both drinking water and feed (flaked corn) sampled at the end of the assay (96 h) were contaminated by fluorescing *E. faecalis*, demonstrating that the flies contaminated these sources with *E. faecalis* throughout the experiment. The role of house flies in the ecology of *E. faecalis* is discussed.

3.2 INTRODUCTION

The house fly, *Musca domestica* (L.) is the most common fly species in the family Muscidae and is distributed worldwide. The house fly is a significant nuisance pest due to high populations and its synanthropic nature. In addition, house flies are recognized as mechanical vectors of a number of parasites/pathogens including protozoans, viruses, fungi and bacteria (Greenberg 1965, Graczyk et al. 2001). A number of house fly attributes contribute to its ability to function as a mechanical vector, namely its feeding habits, abundance and close association with humans as well as the nature of larval developmental sites and dispersal habits. The house fly ingests microbes associated with its food sources and oviposition sites, which can reside transiently in the fly digestive tract, proliferate and can be disseminated through feeding and defecation (Sasaki et al. 2000, Kobayashi et al. 2002). Kobayashi et al. (1999) proposed the term "bioenhanced transmission" to describe this phenomenon, which is more than simple mechanical transmission.

The alimentary canal of the house fly includes a highly modified crop that branches off the esophagus and extends to the abdomen. The crop is a bivalved sac believed to function primarily as a storage organ for sugars, which are utilized largely to support the high energy demands of flight (Singh and Judd 1966). The crop of the house fly has been observed as an important location of bacterial accumulation and proliferation (Kobayashi et al. 1999, Sasaki et al. 2000, McGaughey and Nayduch 2009). Moreover, the crop is significant due to the method by which the fly feeds. Contents of the crop are partially regurgitated with salivary secretions to break down food into a liquid that can be easily drawn in through the sponging mouthparts.

Therefore, bacteria in the crop are easily deposited onto various substrates including human food, drinks and cooking utensils (Graczyk et al. 2001, McGaughey and Nayduch 2009).

Due to the ability of various microbes/pathogens to proliferate in the house fly digestive tract, laboratory studies have focused specifically on tracking the fate of select bacteria in the fly gut. Kobayashi et al. (1999) fed adult flies trypticase soy broth with two strains of *E. coli* O157:H7 at a concentration of 10^9 CFU/ml. Within 1 h of exposure the flies were excreting the bacteria and 10^6-10^7 CFU/fly were recovered from the alimentary canal. The flies continued to harbor *E. coli* O157:H7 for up to 72 h. Additionally, they successfully contaminated their substrates with 10^7 at 1 h down to 10^2 CFU/fly at 72 h. The authors reported evidence of the bacteria actively proliferating on the labellum and in the crop (Kobayashi et al. 1999). A study involving the persistence and transmission of *Aeromonas caviae* by house flies was conducted by Nayduch et al. (2002). In this study, the flies were fed 1.2 x 10^4 CFU of the bacterium suspended in saline and were sampled at multiple intervals during the first 24 h and daily thereafter for up to 12 days. The counts of *A. caviae* recovered from the midgut consistently increased to a peak of 8.9 x 10^4 at 48 h, indicating active proliferation in the fly alimentary canal. From day 2-8 the CFU counts were significantly lower and none were recovered after day 8. Furthermore, groups of six house flies were exposed to ground chicken and actively contaminated the meat from day 1-7 of the experiment. McGaughey and Dayduch (2009) conducted a series of lab experiments to observe the fate of *Aeromonas caviae* in *M. domestica*. Two strains (motile and non-motile) of GFP-producing *A. caviae* were fed to the flies. Viable bacteria were observed in the crop as well as the midgut at 0-22 h. Bacterial counts from the flies at 2 h post infection resulted in a 1000 fold increase of the bacterium, indicating active proliferation in the alimentary canal. The bacteria were lysed in the posterior midgut and no

viable cells were recovered in excreta. By 24 h, no bacteria were observed in the flies, however, the flies were not fed after bacterial infection and the authors postulated that the bacteria would have survived longer had the flies been actively feeding (McGaughey and Dayduch 2009). In fact, in the previously described assay (Nayduch et al. 2002) involving *A. caviae*, the flies were fed following exposure and the bacteria were recovered up to 8 days. These studies underscore the potential role of the house fly as a vector of pathogenic bacteria. It was observed that the bacteria survive transiently in the fly alimentary canal, actively proliferate and are frequently deposited in vomitus and in some cases in excreta as well.

Enterococcus faecalis is a commensal in the digestive tract of several animals including humans. Enterococci are the third most important bacterial group responsible for nosocomial infections and *E. faecalis* causes the majority of infections (Tannock and Cook 2002, Fisher and Phillips 2009). Furthermore, *E. faecalis* frequently harbors a variety of antibiotic resistance genes and is capable of inter and intraspecific gene transfer (Huycke et al. 1998, Fisher and Phillips 2009).

A number of studies have specifically involved the association of *M. domestica* and enterococci. Graham et al. (2009) were interested in the potential role of house flies to acquire and transfer antibiotic resistant (AR) enterococci from broiler poultry operations. They isolated and characterized enterococci from poultry litter at three operations and from house flies both onsite and up to 3.2 km away in a tristate region (Delaware, Maryland and Virginia). The genotypic and phenotypic AR profiles of enterococci from litter and flies matched well, suggesting that the fly plays a role in the ecology and dissemination of enterococci and associated resistance genes at these operations (Graham et al. 2009). Enterococci were also characterized from house flies, German cockroaches and swine feces at two swine operations in

Kansas and North Carolina (Ahmad et al. 2011). Ninety four percent of house flies were positive

for enterococci with a mean concentration of 10^4 CFU/fly. *E. faecalis* was the most abundant

species recovered from flies and *E. hirae* was most common from swine feces. Both sources

(flies and swine feces) had similar phenotypic and genotypic AR profiles as well as AR genes

and mobile genetic elements. Genotyping of *E. faecalis* and *E. faecium* isolates with pulsed-field

gel electrophoresis (PFGE) revealed clonal matches among the bacteria from feces, flies and

roaches. Another study assessed the AR profiles of enterococci from house flies captured in

rural and urban environments as part of an effort to assess fly migration up to 124 km to and

from Manhattan, KS (Chakrabarti et al. 2010). Over 90% of the flies were positive for

enterococci and the AR prevalence among the bacteria was highest from flies captured at cattle

feedlots followed by dairies and urban sites such as restaurants. There was a positive association

of AR prevalence of enterococci from *M. domestica* to distance of restaurants at which the flies

were captured from feedlots. Thus, the authors attributed the feedlots as the primary source of

AR enterococci. Macovei and Zurek (2006) sampled the digestive tracts of *M. domestica* for

enterococci at five fast-food restaurants in Northeastern Kansas. Ninety seven percent of the

flies were positive for enterococci with a mean CFU of 3.1×10^3/fly. Two hundred five of the

enterococcal isolates cultured were characterized. *E. faecalis* was the most abundant species

(88.2%) and AR phenotypic tests revealed resistance to tetracycline (66.3% of isolates),

erythromycin (23.8%), streptomycin (11.6%) ciprofloxacin (9.9%) and kanamycin (8.3%)

(Macovei and Zurek 2006). A follow-up study found food from the same restaurants was

commonly contaminated with AR enterococci (Macovei and Zurek 2007). Three ready-to-eat

food items (chicken salad, chicken burger, and carrot cake) were sampled in summer and winter.

Overall concentration of enterococci throughout the year averaged 10^3 CFU/g with greater

prevalence during the summer (92.0% of salads, 64.0% of burgers) than the winter (64.0% of salads, 24.0% of burger sample). The higher prevalence of enterococcal contamination among food samples in the summer correlates with higher house fly activity, thus indirectly implicating the fly as at least a partial source of contamination. This study implied that food served in restaurants is commonly contaminated with AR enterococci and that flies may play a role in this contamination (Macovei and Zurek 2007). Another study directly assessed the ability of *M. domestica* to contaminate ready-to-eat food with enterococci under laboratory conditions (Macovei et al. 2008). The authors observed that as few as five flies in 30 minutes (the fewest flies and shortest time tested) would result in an average of 3.1×10^3 enterococci deposited on the food.

An important aspect of understanding the role of the house flies in the ecology of *E. faecalis* is determining the ability of the fly to ingest and harbor *E. faecalis*. The aim of this study was to track the fate of *E. faecalis* within the house fly digestive tract for up to 96 h in flies that maintained their acquired gut microbiota and continually gained an influx of microbes from a natural food source.

The objective and hypothesis for this study were presented in Chapter 1 as Objective 2:

Objective 2: Determine the spatial and temporal fate of *E. faecalis* OG1RF in the house fly digestive tract.

Hypothesis: *Enterococcus faecalis* remains viable and multiplies in the house fly digestive tract.

3.3 MATERIALS AND METHODS

3.3.1 House flies

House flies used for the study were obtained from the laboratory colony, Department of Entomology, Kansas State University. The colony was maintained at 25 ± 2°C, $70 \pm 10\%$ relative humidity, and an 18 h light, 6 h dark cycle.

3.3.2 Enterococcus faecalis

Enterococcus faecalis strain OG1RF with plasmid pMV158GFP, with green fluorescent protein (GFP) was used in the assays (Nieto and Espinosa 2003). The strain was maintained on trypticase soy broth agar (TSB; BD, Sparks, MD) and streaked to fresh TSB agar plates, incubated at 37°C for 24 h prior to use.

3.3.3 Assay

Two to five day old house flies were starved for 12 h prior to use in the assays. The flies were then placed individually into 60 x 15 mm petri dishes with a 4 μl solution of sterile phosphate buffered saline (PBS) with the GFP-producing *E. faecalis*. The concentration of the bacterium in the PBS was 3.1 to 7.8 x 10^6 CFU. Control flies were placed in dishes with 4 μl of sterile PBS alone. The flies were observed to verify uptake of the solution for 20 min, then were transferred individually to 60 x 15 mm petri dishes with 0.2 g of cracked corn and 500 μl of sterile tap water. The corn was intentionally not sterilized to mimic field conditions and preserve inflow of microbes from the food source. The flies were maintained at 25 ± 2°C during the assay. Every 24 hours, flies were moved to fresh petri dishes with fresh water and food to reduce continual self-contamination and excessive proliferation with GFP-producing *E. faecalis* on the flies substrate and food source.

Three treated flies were randomly selected for dissection at each time interval (1, 4, 8, 24, 48, 72 and 96 h) and placed in 0ºC for 5 minutes prior to dissection. One control fly was also randomly selected for dissection at all time intervals excluding 4 h due to constraints of handling time. The labellum was first removed and placed in sterile PBS. The fly was then surface sterilized with 0.05 sodium hypochlorite and 70% ethanol for 1 min in each solution. The flies were dissected to access regions of the digestive tract (foregut, midgut, hindgut), which were removed and placed individually in sterile PBS. Each alimentary canal region was homogenized, 1/10 serially diluted, and 100 µl solutions were spread plated on TSB agar plates. All plates were incubated at 37ºC for 24 h. Following incubation, the number of fluorescing colonies was counted to obtain a colony forming unit (CFU) estimate for the respective digestive tract region. The assay was conducted twice resulting in 6 treatment replicates (n=6) for each time period.

To determine the contamination of food and water at the end of the assay (96 h), four samples of water (100 µl) and corn (100 µl from 10 ml solution of H_2O and 0.2 g corn sample) that the flies had utilized during the assay in the petri dishes were sampled and spread onto TSB agar plates and incubated at 37 ºC as described above to determine the presence of the GFP-labeled *E. faecalis*.

The fly digestive tract for each time interval was viewed under a dissecting scope with UV light (Model Nikon SMZ 1500; UV filter, Ex 470/40, dm 495, ba 525/50) (Nikon Corporation, Tokyo, Japan). Observations regarding relative fluorescence were noted and comparisons made with control flies to compare background tissue fluorescence to that resulting from the GFP-expressing *E. faecalis*.

Photographs were taken using camera model Leica DFC 400 with dissecting scope Lecia M205 FA and GFP2 filter (ex 460-500 nm, dm 510 pl) (Leica Microsystems AG, Wetzlar, Germany). Images were enhanced by adjusting contrast and brightness in the software Canvas 9 Professional Edition (ACD Systems International Inc., Victoria, British Columbia, Canada). No other enhancements/alterations were made to photographs.

3.3.4 Statistical analysis

Analysis of variance (ANOVA) of CFU counts for each digestive tract region across the time intervals was performed using SAS (PROC GLM, SAS Institute 1999). Counts were log transformed ($\log_{10}$) to meet assumptions of equal variance. If ANOVA revealed significantly different ($P < 0.05$) counts for a digestive tract region, pairwise comparisons were conducted using the least significant difference (LSD) method in SAS to assign groupings. A comparison of male to female fly mean CFU counts for each gut region was done using ANOVA in SAS.

3.4 RESULTS

Colony forming unit (CFU) counts of *E. faecalis* are presented in Figures 3.1 and 3.2. Viable *E. faecalis* were recovered from at least one region of the fly digestive tract at every time sampled. Statistical analysis of mean CFU per time period revealed significant differences for the foregut ($F = 2.37$; df = 13, 28; $P = 0.0273$) and midgut ($F = 3.0$; df = 13, 28; $P = 0.0072$) while labellum and hindgut CFU counts were not significantly different. Labellum counts were relatively low at 1, 4, 8 and 24 h and were generally higher at 48, 72 and 96 h (Fig. 3.2). Though significantly different, foregut CFU counts were sporadic ranging from $9.4 \pm 6.2 \times 10^3$ at 24 h to $1.8 \pm 0.8 \times 10^6$ at 72 h and increased during the latter part of the assay. The highest midgut CFU count ($2.8 \pm 1.3 \times 10^6$) was obtained at 1 h and declined at 4, 8 and 24 h to a mean

of $9.8 \pm 4.0 \times 10^4$. The CFU count remained relatively constant at 48 h, rose to a mean of $1.3 \pm 1.1 \times 10^6$ at 72 h and fell to $1.7 \pm 1.3 \times 10^4$ at 96 h (Fig. 3.1). Mean hindgut CFU counts were sporadic, ranging from $2.0 \pm 1.1 \times 10^3$ at 24 h to $2.5 \pm 1.2 \times 10^5$ at 72 h and no apparent trends were observed across the time periods (Fig. 3.1). However, it was noted that hindgut counts were consistently lower than the foregut and midgut counts. Mean CFU counts were significantly higher in male over females flies in all regions of the digestive tract; foregut ($F = 10.07$; df $= 1$; $P = 0.0036$), midgut ($F = 9.52$; df $= 1$; $P = 0.0045$), and hindgut ($F = 10.63$; df $= 1$; $P = 0.0029$) and not significantly different by sex among labellum counts. Three samples of drinking water and corn were sampled at the end of the assay at 96 h and resulted in CFU counts of water and corn of $5.5 \pm 4.6 \times 10^5$ and $1.8 \pm 1.0 \times 10^4$, respectively.

Generally, only a dim glow of fluorescence was observed from the foregut and midgut of the flies for the first 24 h of the assay and even at the earliest time of 1 h. However, beginning at 48 h and peaking at 72 h, several of the flies exhibited a marked increase of fluorescence, mostly associated with the crop (Fig. 3.3). At 72 h, the fluorescing bacteria could be observed in the crop lumen, along the duct of the crop and into the midgut. Fluorescence in the midgut was generally greater in the anterior portion and declined until it was no longer detectable in the posterior midgut. Comparison of treatment and control flies revealed that the hindgut exhibited a significant amount of autofluorescence. Therefore, limited observational data could be obtained for this region of the digestive tract.

3.5 DISCUSSION

Better understanding of the fate of bacteria in the house fly alimentary canal is important when considering the fly's role in microbial ecology. A number of recent studies have highlighted the efficiency at which house flies can acquire and disperse antibiotic resistant

enterococci (Graham et al. 2009, Chakrabarti et al. 2010, Ahmad et al. 2011). Further, the ability of various microbes to proliferate and possibly spread antibiotic resistance genes horizontally in the house fly digestive tract, underscores the potential of house flies to amplify bacterial pathogens (Kobayashi et al. 1999, Sasaki et al. 2000, Kobayashi et al. 2002, Petridis et al. 2006, Akhtar et al. 2009, McGaughey and Nayduch 2009). This present study adds to the understanding of the fate of *E. feacalis*, an important nosocomial pathogen common in both clinical and agricultural environments, in the house fly digestive tract.

Midgut counts trended as would be expected from higher to lower for the first 24 h of the assay. Having been starved for 12 h, the flies were likely dehydrated and readily ingested the saline solution, most of which presumably moved directly to the midgut resulting in the initial high counts. The subsequent reduction in *E. faecalis* counts during the first 24 h was likely the result of lyses and digestion of bacteria in the midgut. The hindgut CFU counts were lower relative to the foregut and midgut counts throughout the study, further suggesting that the fly digested the bacterium. This trend was also observed when viewing the digestive tract under UV light; fluorescing bacteria were more apparent in the anterior portion of the midgut as compared with the posterior midgut. The pattern of CFU counts in the foregut was less predictive than midgut, but the relatively higher counts in the latter portion of the assay corresponds with the observation of greater fluorescence seen in the crop. It is likely that some of the bacteria initially ingested were shunted to the crop. After 48 to 72 h the bacteria likely proliferated to the extent that it could be easily viewed in the crop lumen and resulted in the granular pockets of fluorescence observed (Fig. 3.3). Whereas the initial high counts observed primarily in the midgut were due to the initial influx of the inoculum, higher counts, in some cases in the range of 10^6 CFU bacteria, later on in the assay are likely the result of proliferation in the crop.

Furthermore, it is suspected that the bacteria that multiplied in the crop were periodically moved to the midgut as well as regurgitated on food substrates and led to the majority of the *E. faecalis* recovered in the labellum, midgut and hindgut following the first 24 h. Additionally, while the relative counts in the midgut and hindgut were lower at 96 h, the foregut retained a mean CFU count of $7.2 \pm 7.0 \times 10^5$, suggesting that the bacterium continued to proliferate in the crop/foregut. Kobayashi et al. (1999) found the pseudotracheae of the labellum to be important for proliferation of *E. coli* O157: H7. Counts of *E. faecalis* from the labellum in our study were variable but did reveal a trend of higher counts during the latter part of the assay indicating that either the *E. faecalis* multiplied on the surface of the labellum, or propagated in the foregut/crop and were regurgitated during feeding leading to the elevated counts in the labellum after the first 24 h.

The CFU counts were marked by considerable variation. Though a number of variables can influence this, likely the most important contributor was diverse amounts of inoculum initially ingested by individual flies. On visual observation during fly exposure to the inoculum, it was noted that some flies ingested all of the 4 µl of solution while others ingested lesser amounts leaving some in the dish unconsumed. This varying amount of consumed inoculum likely had a substantial impact on bacteria ingested and subsequently the amount available to recover.

Another likely factor contributing to CFU variance involved the relative amount of subsequent feeding and gut activity of individual flies following uptake of the inoculum. Sasaki et al. (2000) noted that female house flies, particularly while eggs were still maturing, fed significantly more often. This is reasonable in light of the increased nutritional requirements associated with egg production. Further, they also observed overall fewer bacteria in the crop of

females and attributed it to more frequent emptying of the crop contents through oral excretion/feeding. Our results support this observation based on female flies having significantly fewer *E. faecalis* in the foregut, midgut and hindgut compared with males at the same time periods.

The experiment was designed to allow interaction between *E. faecalis* and the fly's gut microbiota. Therefore, no attempts were made to modify or reduce the resident fly gut microbes and the flies were provided a food (non-sterile) and water source throughout the assay to better estimate how the *E. faecalis* population would respond with a steady incoming nutritional source. Further, it was expected that the flies would digest more bacteria if starved thereby artificially lowering the bacterial population in the gut compared with flies allowed to feed. Additionally, the food source (cracked corn from a feedlot) was intentionally not sterilized to maintain an influx of associated microbes and allowing interaction and competition with *E. faecalis*.

Due to the presence of abundant food and water and a confined space during the assay, *E. feacalis* were likely deposited on all these substrates and the fly likely reacquired the bacteria during grooming and feeding. In fact, direct evidence of food and water contamination was observed up until the end of the assay (96 h). Therefore, the possibility exists that *E. faecalis* proliferated on the flaked corn and was re-consumed by the fly, thereby inflating the CFU counts observed in the fly gut. Though it is expected that the bacterium did proliferate to some extent on the food source, the majority of proliferation likely occurred in the crop. To lessen the extent of reintroduction of *E. faecalis* over the course of the study, the flies were moved to new plates daily with fresh food and water. Therefore, 24 h would have been the extent of time available for *E. faecalis* to multiply in the dish. If this had been the major contributor to *E. faecalis* in the

fly alimentary canal, a much more consistent CFU count would be expected across the times flies were sampled. Further, the highest CFU counts and observed fluorescence in the fly digestive tract would be expected during the first 24 h. As has been pointed out, with the exception of the midgut, higher counts were generally observed after the first 24 h and across the three gut regions (foregut, midgut, hindgut), the highest combined CFU counts occurred at 72 h (Fig. 3.1). These observations are more consistent with *E. faecalis* proliferation in the gut over propagation only in the food or water sources.

Only fluorescing colonies were counted when taking CFU counts on TSB media. This was necessary because preliminary screening of the colony house flies revealed that some were positive for tetracycline-resistant enterococci. Therefore, even using an enterococci selective media with tetracycline (tetracycline resistance was another marker in *E. faecalis* OGR1F:pMV158) added would have potentially lead to inflated CFU counts. On the other hand, it should be noted that there is a potential for loss of the pMV158GFP plasmid/fluorescence among the bacterial strain (L. E. Hancock, personal communication). This would result in the original GFP-producing *E. faecalis* strain being retrieved from the fly gut that would be indistinguishable from other species of bacteria due to loss of fluorescence. No direct study of pMV158GFP stability has been done for *E. faecalis*, however, Lakticova et al. (2006) used the same plasmid in *E. faecium* D344SRF and tracked its fate in the mouse digestive tract. They found the plasmid was quite unstable in this environment with only 1% of viable *E. faecium* D344SRF in the feces fluorescing. Estimates were not made of the rate of plasmid loss in the present study, however, it was likely not as dramatic as 99% observed by Lakticova et al. based on the relatively high CFU counts observed throughout the study. The potential of *E. faecalis* to

lose the plasmid and subsequent fluorescence over the 96 h of the assay is worth noting and possibly led to underestimates of the actual *E. faecalis* OG1RF population.

This study has implications regarding the role of house flies in the ecology of this medically significant bacterium. Due to the ability of the house fly to disperse up to 12 km (Quarterman et al. 1954), the fly could acquire virulent and antibiotic resistant *E. faecalis* and successfully deposit the bacteria to a number of substrates (human food/drinks) remote from the area they were acquired. Results from this study suggest the need for field experiments to better assess the ability of wild *M. domestica* to disperse and disseminate *E. faecalis* in a natural setting.

In conclusion, house flies serve as a bioenhanced vector of *E. faecalis* under laboratory conditions. Viable *E. faecalis* were recovered from at least one region of the digestive tract of all flies exposed to *E. faecalis* throughout the assay up to the end of the study at 96 h. The flies contaminated their food and water with *E. faecalis* at 96 h following ingestion. The crop of the foregut, as in similar studies utilizing gram negative bacteria, appears to be an important site for proliferation of *E. faecalis*. *E. faecalis* counts were generally higher in male versus female flies, likely due to the higher nutritional demand of female flies for egg production, leading to increased feeding and digestion.

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3.7 FIGURES AND TABLES

Figure 3.1 Mean CFU + SEM of GFP-expressing *E. faecalis* retrieved from the foregut, midgut and hindgut of house flies (n=6 for each time period).
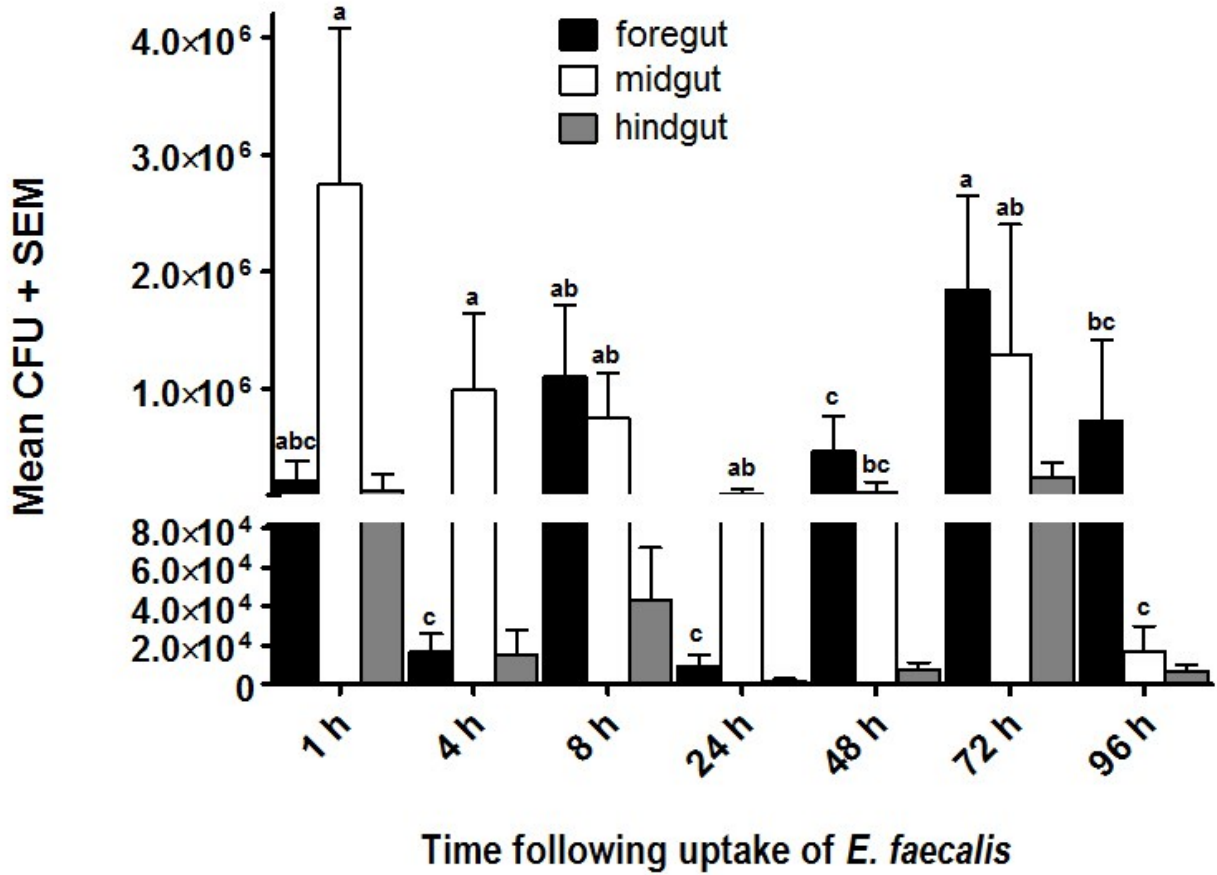
Figure 3.2 Mean CFU + SEM of GFP-expressing *E. faecalis* retrieved from the labellum of house flies (n=6 for each time period).
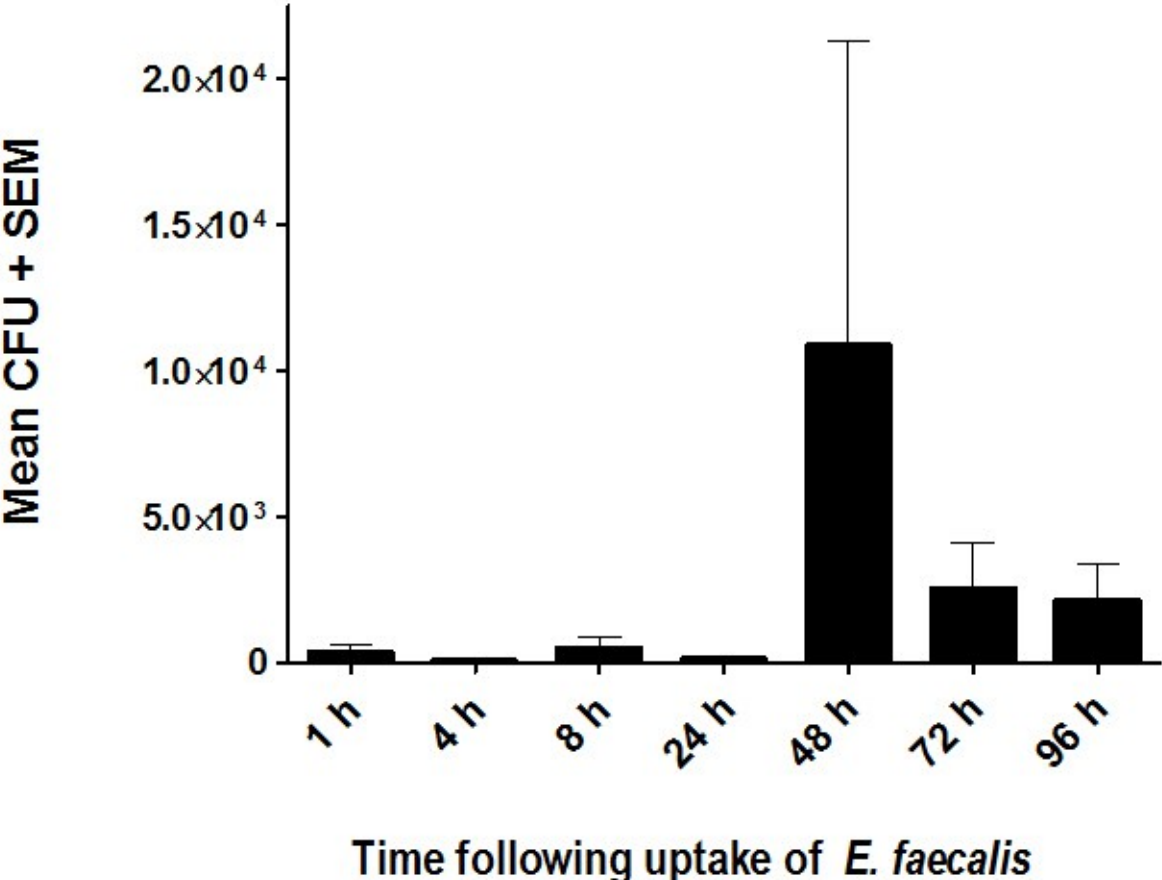
Figure 3.3 GFP-expressing *E. faecalis* in house fly digestive tract at 72 h following ingestion. A. Crop, midgut (MG) and peritrophic matrix (PM) exhibiting granular pockets of fluorescing bacteria. Scale bar, 1 mm. B. Control crop and three treatment crops (T1, T2, T3). Variation in fluorescence and crop distention are evident among the three treatments. Scale bar, 1 mm.
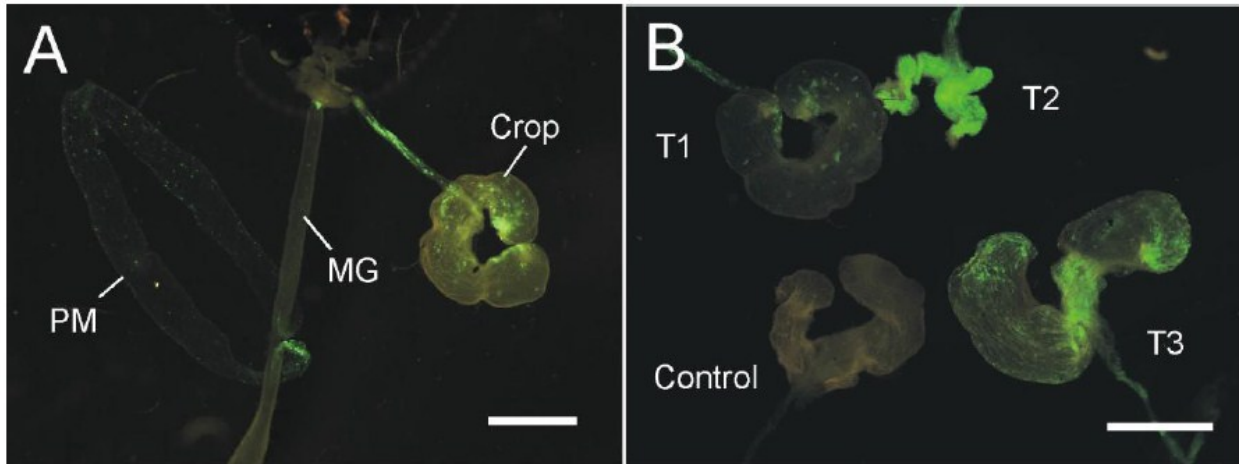
CHAPTER 4: DEWATERED SEWAGE BIOSOLIDS PROVIDE A PRODUCTIVE LARVAL HABITAT FOR STABLE FLIES AND HOUSE FLIES

4.1 ABSTRACT

Dewatered biosolids (biosolid cake) stored at a wastewater treatment facility supported larval development of numerous Diptera. A study was conducted during 2009 and 2010 to assess the diversity of calyptrate muscoid flies developing in the biosolids and track their seasonal abundance by use of emergence traps. Traps were employed at the site for a total of 47 weeks; 22 weeks in 2009 (19 May-20 Oct) and 25 weeks in 2010 (27 May-18 Nov) totaling 386 trap-weeks. A total of 11,349 calyptrate muscoid flies were identified and counted. The three most common species were stable flies (*Stomoxys calcitrans*) (9,016, 80.2%), house flies (*Musca domestica*) (2022, 18.0%) and calliphorid flies (289, 2.6%). Numbers of stable flies and house flies peaked around mid-July each year and a second, smaller peak was observed for stable flies 5-8 weeks later. Estimated annual emergence of for stable flies was 551,404 and for house flies 109,188 and overall fly production was estimated at 682 stable flies/m^2 and 135 house flies/m^2. This study provides new insights in to the utility of biosolid cake as a larval development habitat for stable flies and house flies.

4.2 INTRODUCTION

Stable flies, *Stomoxys calcitrans* (L.) (Diptera:Muscidae) are blood feeders of livestock and other animals and the greatest pest of cattle in the US. Effects on cattle from stable flies include triggering behavior modifications such as cattle bunching and standing in ponds to avoid bites, which reduces feeding and weight gain/milk production (Campbell et al. 2001). The economic impact of stable flies on the US cattle industry has been estimated at $1 billion annually (Taylor and Berkebile 2006).

The house fly, *Musca domestica* (L.) (Diptera: Muscidae) is an abundant and ubiquitous pest of humans and livestock. House flies do not bite or take bloodmeals but have sponging mouthparts and feed on a wide range of organic material (Pratt et al. 1975, Iwasa 1983). House flies are a significant nuisance pest due to the high populations and their synanthropic nature. Further, they are recognized as mechanical vectors of a number of parasites/pathogens including protozoans, viruses and bacteria (Greenberg 1965, Graczyk et al. 2001).

Stable flies and house flies develop as larvae in a variety of decaying organic matter such as human and animal feces, rotting vegetation, silage and garbage. An active microbial community in the larval substrate is necessary for proper development of both flies (Zurek et al. 2000, Romero et al. 2006). Stable flies commonly utilize aged (14-21 d) cattle manure for larval development in areas such feedlots and dairies (Meyer and Peterson 1983, Skoda et al. 1991, Broce and Haas 1999). Additionally, the advent of large round bale hay feeders used for winter feeding of pastured cattle has provided a prime larval development site for stable flies on pastures (Hall et al. 1982, Broce et al. 2005, Talley et al. 2009, Taylor and Berkebile 2011). These sites serve as an accumulation point for cattle where manure, wasted hay, and urine mix

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with the soil to create an ideal habitat for larval development the following spring. Common developmental sites of stable flies in urban settings include compost piles (Broce 1993). House flies are more flexible in their choice of larval substrates and have been found in poultry manure (Stafford and Bay 1987), fresh as well as aged cattle manure (Meyer and Peterson 1983, Broce and Haas 1999), horse manure, garbage, human feces (Greenburg 1959), carrion and rotting vegetation (Keiding 1986). Stable fly larval development can be as short as eight days (Parr 1962) and five days for house flies (Pratt et al. 1975, Lysyk and Axtell 1987) under ideal conditions.

A 2007 report from the North East Biosolids and Residuals Association (NEBRA) estimated that there were approximately 17,000 operating wastewater treatment facilities (WWTF) in the US (NEBRA 2007). Two primary products are generated during wastewater processing, disinfected effluent and treated biosolids. Effluent is generally released to natural bodies of water such as lakes and rivers. Approximately 40-60% of biosolids produced in the US are used to fertilize agricultural land; other forms of disposal include incineration and landfill application (Krauss and Page 1997, National Research Council 2002, Arthurson 2008). Stabilized biosolids contain approximately 5-7% solids, making them a thick liquid that is usually pumped into and stored in closed-contained bins before being transported to final disposal. Among the 135 WWTF in Kansas, approximately 75% of operations dewater their biosolids by equipment such as a belt press for open air storage (M. Gerard, Kansas Department of Health and Environment, personal communication). Belt pressing increases the solid content to approximately 20% and thickens the biosolids to a consistency known as biosolid cake, which can be transported and maneuvered with equipment such as front end loaders and dump trucks (Forster 1985). Biosolids that are processed in this manner provide a rich organic substrate that

is of suitable moisture content for development of several filth fly larvae (Dadour and Voss 2009).

Dadour and Voss (2009) conducted a year-long study of the utilization of biosolid cake by muscoid flies in Perth, Australia. House flies and stable flies were the most abundant flies emerging from the biosolids, accounting for 97% of all species. The authors measured six parameters of the solids relative to their utilization by the flies; ambient temperature, moisture, rainfall, ammonium concentration (NH_4), pH and age. Three of these, biosolid age, NH_4 content and ambient temperature were correlated the most with fly oviposition and development.

A small WWTF that served a community of under 5,000 in Northeast Kansas processed biosolid cake as described above. The solids were utilized by applying them to local crop fields in the spring before planting. After planting, no more biosolids could be applied so they were stockpiled in an open area on the property of the wastewater facility from early-middle May until the following spring when they could again be removed and applied to fields. Thus, the biosolids were stored onsite for up to 10 months and accumulated throughout this period. While the solids were stored, several adult flies including psycodid moth flies, stable flies and house flies were attracted to the substrate and larvae, presumably of the same flies, were observed developing in it. A study was conducted during 2009 and 2010 to identify and monitor seasonally flies emerging from the biosolids.

The objective and hypothesis for this study were presented in Chapter 1 as Objective 3:

Objective 3: Assess biosludge at a wastewater treatment facility as a larval developmental habitat of muscoid flies.

Hypothesis: Biosludge at wastewater treatment facilities can serve as a developmental habitat for muscoid flies.

4.3 MNMATERIALS AND METHODS

4.3.1 Study site

The wastewater treatment facility involved in this study (Site 4 from Chapter 2) received 450,000 gallons of waste daily from a population of 4,400. The facility utilized activated sludge to process liquids and aerobic digestion of solids (sludge) for approximately 20 days (Cheremisinoff 1996, Horan 1990). Following sludge digestion, the stabilized biosolids were dewatered by use of a belt press and stored in an open field onsite (Fig. 4.1). Biosolids were deposited 1-2 times weekly with a dump truck and accumulated from approximately the middle of May until the following spring when they were removed and land applied to agricultural fields. The weekly rate of biosolid deposition was 23-35 m^2 of surface area and averaged 0.5 m deep.

4.3.2 Sampling of flies

Fly trapping was initiated in the spring during 2009 and 2010 after the stockpiled solids had been removed for land application and fresh biosolids began being deposited onsite (Fig. 4.1). Emergence traps were positioned on the newly deposited material and the study continued throughout the season until no stable flies or house flies were captured in any of the traps. Traps were employed at the study site for a total of 47 weeks over the two-year study; 22 weeks in 2009 (19 May-20 Oct) and 25 weeks in 2010 (27 May-18 Nov) totaling 386 trap-weeks.

Flies were sampled with pyramid emergence traps (Broce and Haas 1999). The base of the trap was constructed of a square wooden frame with a quadrilateral pyramid shaped wire screen rising up 76 cm above the base, which functioned to isolate a ¼ m^2 area of the surface it was placed on (Fig. 4.2). Adult flies emerging from the substrate below the trap move upwards along the screen and enter the 470 ml plastic collection cup attached to the trap apex (Fig 4.2).

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Traps were placed along the margins of the biosolid deposits and were left in place for 3 weeks. Following the 3 week interval, traps were repositioned to another site on the same aged deposit. Traps were placed firmly down on the solids to prevent entry of adult flies from around the base of the trap. The assumption was that any flies trapped were the result of oviposition before trap placement and larval development in the area below the trap. Traps were sampled at ≤ 7 day intervals by removing the collection cup; immediately a new cup was placed on the trap to reset it. Collection cups were transported to the laboratory and placed in the freezer until contents were identified and counted. Trap results were calculated as mean flies/trap for the respective period between trap set and retrieval. Biosolids accumulated throughout the season and were deposited approximately weekly. Traps were not placed on fresh deposits in order to allow (1-4 weeks) for flies to oviposit on the material before trap placement, which excluded any further oviposition.

During the 2009 study, six traps were initially placed and 4 more were added (29 Jun – 10 Aug) as fly activity increased and more solids were deposited. By the end of the season the traps were reduced back down to a total of 6 (1 Sep – 20 Oct). As fly counts diminished in aging biosolids, traps were moved to more recently deposited solids throughout the study. The area of biosolids during 2009 increased from 148 m^2 on 19 May to 920 m^2 by 20 Oct. The mean area of the biosolids directly sampled with emergence traps during 2009 was 2.1 m^2 (range 1.5 – 2.5 m^2) and percent of total biosolids area sampled ranged from 1.0% on 19-25 May to 0.2% during 13-20 Oct. During 2010, the number of traps placed ranged from 4 during the beginning of the study, increased up to 11 during 12–19 Aug and down to 3 by 11 Nov. The total area of biosolids increased from 177 m^2 on 27 May to 697 m^2 on 11 Nov. The mean area of biosolids

directly sampled with emergence traps during 2010 was 2.1 m^2 (range 0.75 – 2.8 m^2) and percent of total biosolid area sampled ranged from 0.6% on 27 May – 3 Jun to 0.1% during 11-18 Nov.

4.3.3 Identification of flies

Assistance with identification of the Phycodidae flies collected was provided by Dr. Greg Zolnerowich, systematist and curator of the Kansas State University Museum of Entomological and Prairie Arthropod Research. Flies other than house flies, stable flies or calliphorid flies were occasionally captured throughout the study. During the 2010 season, these flies were identified by fly systematist Dr. Jade Savage of Bishop's University, Quebec and voucher specimens (voucher no. 219) were submitted to the KSU Museum of Entomological and Prairie Arthropod Research.

4.3.4 Estimates of total fly emergence

The area of the biosolids was used to estimate the overall fly emergence at the site. This was chosen over other possible measurements such as total mass of material deposited. Provided that the biosolids were of sufficient depth to maintain needed moisture, which was likely the case for the majority of the deposits, the area would have been the limiting factor as the larvae likely utilized the solids within a few cm from the surface rather than utilizing the entire column of material during development. In fact, Taylor and Berkebile (2011) studied the vertical distribution of stable fly larvae in pasture hay feeding sites and found over 90% of the larvae within the top 5 cm of the substrate.

The estimated emergence was determined by multiplying the fly counts from traps by two parameters; (1) the mean amount of new biosolids deposited weekly during the respective season and (2) 9 weeks - the amount of time that the majority of flies emerged from the biosolids before it had aged too much to be suitable for larval development. This 9 week duration is

consistent with observations of Dadour and Voss (2009) where 99.9% of flies emerged during the summer from biosolid cake within the first 10 weeks of aging. The mean area deposited weekly multiplied by 9 (weeks) resulted in an area constant, which was multiplied by the fly count per m^2 for the respective week. For 2009, the area constant was 315 m^2 (35 m^2 x 9 weeks) and for 2010 207 m^2 (23 m^2 x 9 weeks). The mean count of stable flies/house flies per square meter was determined by multiplying the mean trap capture of the observed period by 4 (area of trap was ¼ m^2). This mean count per square meter was then multiplied by the area constant to estimate total stable fly/house fly emergence.

The estimate of stable flies and house flies per square meter of biosolids was calculated by dividing the annual estimated flies emerging from the site by the mean total area of the solids that accumulated each season.

4.3.5 Analysis of trapping data by accumulated degree-days

Accumulated degree-day data was calculated based on hourly ambient temperatures recorded by a cooperative weather station nearby the study site and obtained from the High Plains Regional Climate Center (University of Nebraska, Lincoln, NE). The developmental threshold of 10ºC (DD$_{10}$) was used based on previous research of stable flies and house fly larval development (Lysyk and Axtell 1987, Lysyk 1993) and calculated using a sine wave method as developed by Allen (1976). Because both the data of biosolid deposition and fly emergence occurred within a seven day window, degree-day accumulations were based on a middle date of the respective seven day period.

Three fly developmental parameters were analyzed using accumulated degree-days, one was DD$_{10}$ to first emergence of stable flies and house flies. This estimate was calculated when a trap capture from a recent deposit began at zero and subsequently increased thereafter. These

criteria were met five times for stable flies and seven times for house flies allowing accumulated degree-days to be totaled from the deposition date of the biosolids to the week of first emergence of the respective fly. Another developmental parameter analyzed was degree-days to peak seasonal emergence of stable flies and house flies. This was calculated based on the age of the biosolids that produced the greatest amount of stable flies and house flies during the seasonal peak. The date of biosolid deposit was between 27 May – 3 Jun 2010 and the seasonal peak of both flies was 15-22 Jul, therefore, degree-days from 31 May to 18 Jul were totaled. The final analysis involved estimating the degree-days between the two seasonal peaks of stable fly captures and involved totaling DD_{10} from the week of the first peak to the week of the second peak. Degree-day analysis for calculating first emergence and peak seasonal emergence was only possible for data collected during the 2010 season due to less precise tracking of deposit age and associated traps during 2009. Both years' data were used to calculate degree-days between the two stable fly seasonal peaks.

4.4 RESULTS

4.4.1 Trapping totals

A total of 11,349 calyptrate muscoid flies were identified and counted during the study consisting of 9,016 (80.2%) *S. calcitrans*, 2,022 (18.0%) *M. domestica* and 289 (2.6%) calliphorid flies (family Challiphoridae), and 22 flies occasionally captured of three families (Anthomyiidae, Sarcophagidae, Muscidae). The overall fly/trap rate during the study was 20.1 ± 3.2 for stable flies and 4.7 ± 1.1 house flies (Table 4.1). Psycodid moth flies (*Psycoda* spp) appeared to be the most abundant of all flies based on visual observations, but the traps (mesh size of screening) and periods between trap retrieval (too much decomposition of specimens) were not appropriate for their accurate recovery so were not included in the data. Other flies

were captured occasionally and during 2010 were identified to family or genus.   A total of 27

were captured (< .01% of total capture) and 22 identified, which consisted of 11 Anthomyiidae,

Delia spp.; 8 Sarcophagidae; and 3 Muscidae, *Lispe* spp.

4.4.2 Stable fly seasonal captures

During 2009, a total of 4,247 stable flies were captured (range 0 – 230 flies/trap). Mean

stable fly/trap increased from 9.7 ± 1.7 during 19-26 May to a season peak of 90.1 ± 24.8 during

7-13 Jul and then steadily declined for the next four weeks to 1.6 ± 0.7 from 31 Jul - 7 Aug (Fig.

4.3). A second peak occurred during 25 Aug - 1 Sep at 50.1 ± 34.2 flies/trap followed by a

steady decrease for the remainder of the season and falling to zero after 13 Oct. During 2010, a

total of 4,769 stable flies were captured (range 0 – 989 flies/trap). Mean stable fly/trap increased

from 3.8 ± 2.5 during 27 May – 3 Jun and peaked at 152.3 ± 109.2 during 15-22 Jul, followed by

a sharp decline to 17.3 ± 7.2 the following week (Fig. 4.3). Five weeks following the season

peak, a second, smaller peak was observed during 19-26 Aug at 78.4 ± 38.3 flies/trap, after

which mean captures remained below 25 flies/trap and fell to 1.0 ± 1.0 by the final week of the

trapping season (11-18 Nov) (Fig. 4.3).

4.4.3 House fly seasonal capture

During 2009, a total of 581 house flies were captured (range 0 – 129 flies/trap).   House

fly captures during 2009 were overall lower than that of stable flies but peaked with stable flies

during 7-13 Jul at 22.5 ± 12.8 flies/trap (Fig. 4.3). No second peak of house fly captures

occurred and counts fell to zero after 25 Aug. The majority (532 out of 581, 92%) of house flies

were captured in a five week period from 29 Jun to 31 Jul (Fig. 4.3). During 2010, a total of

1441 house flies were captured (range 0 – 287 flies/trap). House fly captures were below those

of stable flies with the exception of one week (8-15 Jul), where house fly captures were slightly

higher (Fig. 4.3). The majority (1251 out of 1441, 87%) of house flies were captured during a seven week period from 8 Jul to 19 Aug. Captures peaked for the season at 56.8 ± 29.8 flies/trap during 15-22 Jul and declined for the remainder of the season falling to zero after 28 Oct (Fig. 4.3).

4.4.4 Calliphorid captures

Of the 245 blow flies captured in 2009, 241 (98.4%) were captured in a four day period (1-5 Jun) and no blow flies were captured after 22 Jun. In 2010, 44 blow flies were captured with 36 (81.8%) captured between 27 May – 3 Jun and seven of the remaining eight were captured during 15-22 Jul.

4.4.5 Estimated fly emergence

Estimated annual emergence of for stable flies was 551,404 and for house flies 109,188 (Table 4.1). During 2009, the total area of biosolids was 920 m^2 by the end of trapping on 20 Oct and during 2010 the area was 697 m^2 when trapping commenced on 18 Nov. The overall fly production was estimated by dividing the estimated total emergence of stable flies (551,404) and house flies (109,188) by the mean total area of the deposits accumulated annually (808.5 m^2), which resulted in an estimated 682 stable flies/m^2 and 135 house flies/m^2 (Table 4.1).

4.4.6 Degree-day analysis

Three developmental parameters of stable flies and house flies were analyzed using accumulated degree-days with a 10ºC developmental threshold; DD_{10} to first emergence, DD_{10} to peak seasonal emergence and DD_{10} between the two stable fly seasonal peak captures. The mean value to first emergence of stable flies was 325.0 ± 32.0 DD_{10} (range 205-410) and for house flies was 360.9 ± 55.2 DD_{10} (range 204-614). Accumulated degree-days to peak seasonal emergence of stable flies and house flies totaled 714 DD_{10}. The accumulated degree-days

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between the two stable fly seasonal capture peaks for 2009 was 661 DD_{10} and for 2010 was 635 DD_{10}.

4.5 DISCUSSION

Stable flies were the most abundant muscoid fly emerging from the biosolids stored at the WWTF, accounting for 80% of the flies captured followed by house flies at 18%. These two species accounted for 97.8% of the total. These results are consistent with those of Dadour and Voss (2009) regarding relative abundance of stable flies and house flies emerging from biosolid cake in Australia. In their study, a total of 5,303 flies were identified and house flies and stable flies accounted for 97% of the total. However, the proportions of these two differed in our study from that observed by Dadour and Voss where house flies were the most abundant species captured at 58% followed by stable flies at 38%. Perhaps the greater abundance of stable flies in our study was a result of migration of the flies from a nearby cattle feedlot within 2 km of the site, which is well within the dispersal range of stable flies (Jones et al. 1999, Taylor et al. 2010). Blow flies consisted of 2% of the total and their utilization of the biosolids was much shorter (82-92% of the seasonal catch occurring each year during a single week in early June) compared to that of house flies and stable flies.

Captures of both stable flies and house flies displayed an overall similar pattern during the two-year study. Captures of both flies peaked during the second (2009) or third (2010) week of July. In 2009, a second, smaller peak for stable flies occurred eight weeks following the first during 25 Aug - 1 Sep (Fig. 4.3). In 2010, a second stable fly emergence peak occurred five weeks following the first on 19-26 Aug (Fig. 4.3). This bimodal pattern among stable fly populations has been observed by others in other stable fly habitats (cattle pastures and feedlots) (Hall et al. 1983, Lysyk 1993, Broce et al. 2005, Taylor et al. 2007, Taylor and Berkebile 2011).

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The underlying cause of the differences in the duration between peaks between 2009 and 2010 (eight weeks versus five weeks apart, respectively) may be best explained by the effect of temperature on fly development. During 2009, where the greater time between the two peaks was observed, the daily temperatures were consistently lower than those of 2010. In fact, the accumulated degree-days for the two periods were 661 DD_{10} during 2009 (8 weeks) and 635 DD_{10} during 2010 (5 weeks). Therefore, despite the time disparity between the two years, the accumulated degree-days were comparable. In 2009, a greater number of stable flies emerged throughout June as compared to that of 2010. Conversely, in 2010 stable flies were captured at a greater rate past the end of July as compared to that of 2009. Additionally, the flies were captured in 2010 at low levels an additional five weeks beyond what was observed in 2009. Specific causes for these differences are not apparent but may be due to the stochastic population variation.

The overall production of stable flies per square meter was estimated at 682 flies/m^2. This amount of productivity is below the estimates from well known stable fly development sites. Broce et al. (2005) calculated an average of 10,600 stable flies/m^2 and Taylor and Berkebile (2011) estimated 1,581 stable flies/m^2 from pasture sites with round bale hay feeders. Further, counts of larvae have been reported as high as 28,000/m^2 in a diary environment (Patterson and Morgan 1986). The lower fly production observed in the biosolids may indicate that this larval habitat is nutritionally inferior to substrates containing cattle manure. Laboratory assays assessing the overall potential of biosolids to produce stable flies remains to be conducted. Another potential explanation for the lower productivity among biosolids is the distance of this site to known blood meal sources. With the nearest feedlot at 2 km away, the wastewater facility might be less utilized by stable flies due the lack of hosts in the immediate

vicinity. Despite the apparent lower fly production, this site offered a significant amount of material for fly development throughout the season. Given the overall greater surface area of this site over that of typical pasture hay bale feeders sites, the relative contribution of this location to stable fly/house fly populations cannot be marginalized.

It is noteworthy that the seasonal peak of stable fly emergence observed at this site in mid-July occurred later in the season than has been observed from pasture hay feeding sites. Taylor and Berkebile (2011) observed stable fly emergence during 2005-2008 among six sites near Ithaca, NE and noted a peak in emergence near the end of June to early July. Further, Broce et al. (2005) took core samples among nine hay feeding sites near Manhattan, KS and observed the greatest emergence of stable flies during 17-25 May over samples taken 29 May – 7 Jun and 13-25 Jun. This would lead to the prediction of peak fly emergence at this site closer to the end of May as observed in Kansas pastures by Broce et al. (2005). In fact, the peak of emergence in this study was 7-8 weeks later. It must be noted that that while stable flies were likely emerging from pasture hay bale feeding sites and other sites in May, the biosolids at the wastewater plant were being removed and land applied and had just begun to be stored by middle May. Therefore, the substrate at the wastewater facility was not available long enough to support stable fly development and emergence in late May. Additionally, though not assessed directly, there is evidence that an amount of biosolid aging was necessary for optimal stable fly oviposition. Previous research indicates this phenomenon (Dadour and Voss 2009) as do degree-day data from this study, which is addressed below. Further, it is well established that ovipositing stable flies prefer cow manure aged 1-3 weeks (Broce and Haas 1999, Romero et al. 2006). Therefore, given the delayed availability of the biosolids and possible lag in oviposition, the seasonal peaks of stable flies and house flies in mid-July are better understood.

Though a significant number of stable flies are estimated to have emerged from this site, they likely dispersed from this area in search of hosts. Moreover, no stable fly bites were experienced during any of the visits to the site despite the common presence of adult flies. It is likely that the majority of the flies present had either recently emerged and were not yet seeking a blood meal, or were ovipositing fed females. Many of the stable flies likely migrated to a 400-head cattle feedlot that was 2 km southwest of the wastewater facility. Other potential destinations include the community that was served by the WWTF less than 0.5 km away.

House flies captured during both years of this study were largely confined to the month of July in 2009 (92% of flies captured) and July into mid-August during 2010 (87% of flies captured) and peaked during mid-July (Fig. 4.3). Only one emergence peak occurred each year rather than two as observed for stable flies. This observation was unexpected as adult house fly populations generally peak later in the summer and often occur in a bimodal pattern in this region (Pratt et al. 1975). For example, Black and Krafsur (1985) sampled flies throughout the season with sticky traps at an Iowa dairy and pasture and observed a peak in late June – early July and a second, greater peak in late August. It is expected that many of the house flies from this site dispersed as well based on what is known about their behavior (Quarterman et al. 1954 a, 1954b, Chakrabarti et al. 2010).

Accumulated degree-days to first emergence of stable flies and house flies were 325.0 ± 32.0 and 360.9 ± 55.2 DD_{10}, respectively. These values are considerably higher than the expected values of 232 DD_{10} for stable flies and 222 DD_{10} for house flies (Lysyk and Axtell 1987, Lysyk 1993). Further, degree-days to peak seasonal emergence of the two flies was 714 DD_{10}, where Taylor and Berkebile (2011) observed stable fly peak emergence at 419 DD_{10}. This difference might best be explained by delayed oviposition on the biosolid deposits. In

calculating accumulated degree-days, the start date for measuring fly development was the date of biosolid deposition from which the flies emerged. This assumed that fly oviposition occurred immediately after the solids were available. However, in the studies of fly production from biosolid cake by Dadour and Voss (2009), stable fly and house fly emergence was measured from biosolid cake exposed to flies for two to over 40 weeks in two-week intervals. They observed the greatest fly emergence from solids that had been exposed for four weeks over just two weeks, or for greater than four weeks. Therefore, the majority of oviposition likely occurred between 2-4 weeks of biosolid exposure/aging. Considering our study, if degree-day calculations are adjusted for fly oviposition by delaying the beginning date by one week the estimates of stable fly and house fly first emergence are 228.2 ± 30.6 and 264.1 ± 54.6 DD_{10}, respectively and the estimate to peak emergence of the two flies is 619 $DD_{10.}$ These values are much more consistent with previous observations, particularly for degree-days to first emergence. Another characteristic inherent in these data which leads to variability is the imprecision of the known date of biosolid deposition and fly emergence. Because the site was in most cases visited weekly, the dates for these two factors occur within a seven day window and therefore could vary by as much as six days.   With as many as 20 degree-days accumulating during the hottest period of the summer, a few days can make a marked difference on degree-day estimates. Therefore, given the unknown date of fly oviposition and specific dates of biosolid deposition and fly emergence, the degree-day estimates of fly developmental parameters seem to approximate the actual values.

Although the biosolids decreased in attractiveness to flies while aging, the fact that new deposits were routinely added resulted in a relatively constant amount of substrate suitable for fly development throughout the season. This allowed for better assessment of the seasonal effect

over sites such as round bale feeder sites, where manure accumulates only in the winter and the site is abandoned by cattle in the spring when they can forage. These pasture sites tend to be heavily utilized in the spring but lose stable fly productivity by the summer (Broce et al. 2005). Not only was biosolid deposition consistent but it was also of sufficient volume and thickness to help ensure adequate moisture to support larval development for weeks/months. Biosolids accumulated in a dump truck that received them from the belt press and were deposited once the truck bed was full. This resulted in a substantial volume of material added that averaged 0.5 m thick. This thickness likely allowed larvae to adjust their distribution in the material to optimal moisture level as the upper region of the material dried out or as moisture increased with rainfall. This is contrasted with feedlots and dairies where manure management practices are aimed at reducing available larval habitats by frequent manure removal. At these sites, fly development is usually limited to areas where manure both accumulates and is not easily removed such as under fence rows or around and under feed bins. Unlike the stored biosolid cake, the thickness of manure at feedlots and dairies varies and may not be sufficient to maintain moisture long enough to support complete larval development. Therefore, biosolids, though perhaps not the most suitable developmental habitat, offer stable flies a larval substrate of adequate moisture and volume from spring to fall.

This study provides valuable insights into the utility of sewage biosolid cake as a larval development substrate, particularly for stable flies and house flies. The estimated annual emergence was 551,404 stable flies and 109,188 house flies. Further, the potential exists for the flies to carry microbes including pathogens and/or antibiotic resistant bacteria present in the solids as they emerge and disperse. This potential public and veterinary health risk should be

considered by wastewater treatment operators, municipal planners and public health

professionals as they make decisions regarding sewage biosolid storage and disposal.

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4.7 FIGURES AND TABLES

Figure 4.1 Stockpiled dewatered biosolids (biosolid cake) at the wastewater treatment facility. A = west end of field with concrete barrier, B = east end of field with overflow deposits.

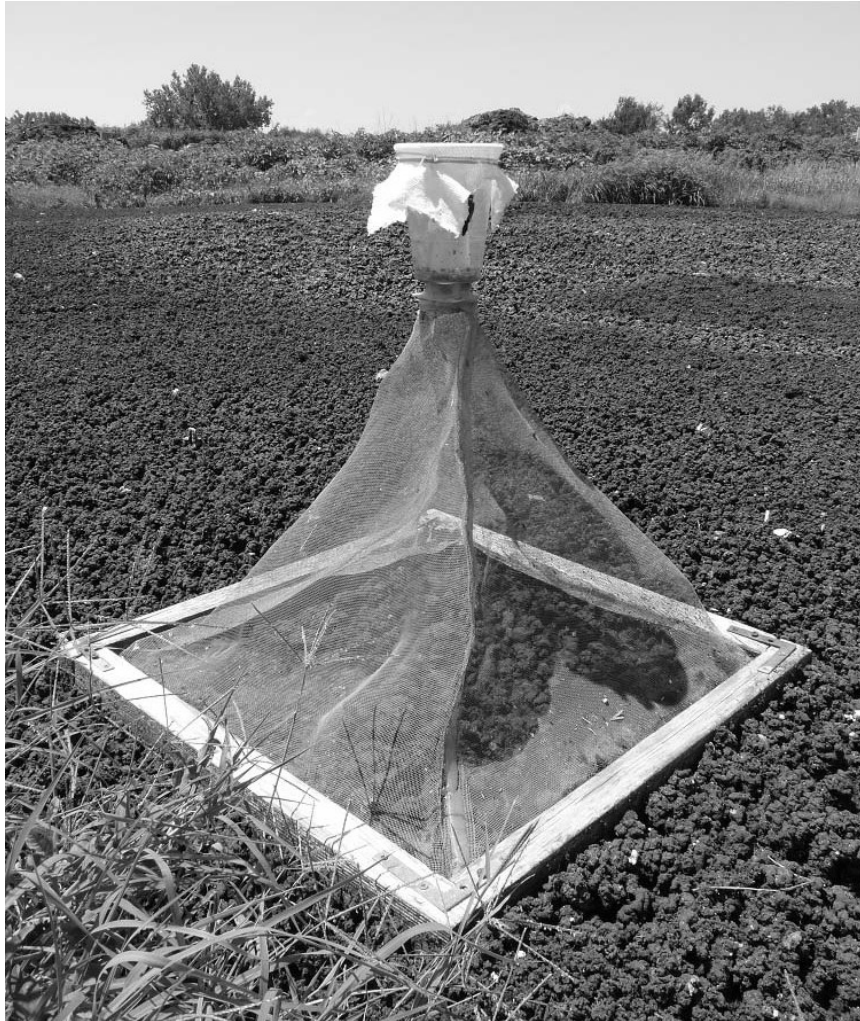**Figure 4.2** Emergence trap with collection cup on dewatered biosolid cake deposit.

Table 4.1 Stable flies and house flies captured and estimated emergence from biosolid cake at a wastewater treatment facility.

	Total captured*	Mean fly/trap ± SEM*	Estimated fly emergence per season	Estimated fly production per m^2
Stable Flies	9,016	20.1 ± 3.2	551,404	682
House Flies	2,022	4.7 ± 1.1	109,188	135

* trapping occurred from 19 May to 20 Oct (2009) and from 27 May to 18 Nov (2010)

Figure 4.3 Stable flies and house flies collected by pyramid emergence traps from dewatered biosolid cake stored at a wastewater treatment plant. A = year 2009, B = year 2010. Trapping occurred from 19 May to 20 Oct (2009) and from 27 May to 18 Nov (2010).