Fate of Antibiotic Resistance Genes During Anaerobic Digestion of Wastewater Solids

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ABSTRACT

Bacterial resistance to antibiotics has become a worldwide health problem, resulting in untreatable infections and escalating healthcare costs. Wastewater treatment plants are a critical point of control between anthropogenic sources of pathogens, antibiotic resistant bacteria (ARBs), antibiotic resistance genes (ARGs), and the environment through discharge of treated effluent and land application of biosolids. Recent studies observing an apparent resuscitation of pathogens and pathogen indicators and the widening realization of the importance of addressing environmental reservoirs of ARGs all lead toward the need for improved understanding of ARG fate and pathogen inactivation kinetics and mechanisms in sludge stabilization technologies.

This research has investigated the fate of two pathogens, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli*, and various ARGs under pasteurization, anaerobic digestion, biosolids storage, and land application conditions. Pathogen die-off occurs at a rate specific to each pathogen and matrix in ambient and mesophilic temperature environments. Viable but nonculturable (VBNC) states are initiated by thermal treatments, such as thermophilic digestion and possibly pasteurization, and allow the persistence of pathogen cells and any ARGs contained therein through treatment and into the receiving environment where resuscitation or transformation could occur.

Raw sludge ARG content does affect digester effluent quality, although the predominant mechanisms of ARG persistence may be different in mesophilic versus thermophilic digestion. In both thermophilic and mesophilic digestion, a correlation was observed between raw sludge and digester ARGs associated with Class 1 integrons, possibly as a result of horizontal gene transfer. ARB survival was shown to contribute to ARG content in mesophilic digestion, but not thermophilic digestion. Thermophilic digestion may achieve a higher ARG reduction because of reduced microbial diversity compared to mesophilic digestion. However, it is evident that horizontal gene transfer still does occur, particularly with highly mobile integrons, so that complete reduction of all ARGs would not be possible with thermophilic digestion alone.

Surprisingly, the experiments that introduced various concentrations of antibiotic sulfamethoxazole and antimicrobial nanosilver did not induce enhanced rates of horizontal gene transfer.

Finally, ARG concentrations in biosolids increased during cold temperature storage suggesting that there is a stress induction of horizontal gene transfer of integron-associated ARGs.

DEDICATION

This work is dedicated to my children: Catlin, Holland, and Baby Miller #3. I hope in some small way this research will contribute toward a healthier and safer environment for you.

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Last, but certainly not least, I would like to acknowledge my friend and husband - Jesse. These last five years have been the most challenging of my life emotionally, physically, and mentally - and school has been tough, too! I could not have done it without our combined blood, sweat, and tears.

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CHAPTER 1

Introduction

Background

Bacterial resistance to antibiotics has become a worldwide health problem, resulting in untreatable infections and escalating healthcare costs. The connection between antibiotic abuse in medical and agricultural arenas with antibiotic resistance is well established, although the prevention of continued spread of resistance is less clear. Pruden et al. (2006) identified antibiotic resistance genes as an emerging contaminant in the environment. Indeed, addressing the environmental reservoirs of genetic elements encoding antibiotic resistance may be part of the solution to abate or attenuate the spread of resistance between clinically-relevant microorganisms and environmental microorganisms (Singer, et al. 2006; Davison, 1999; Wellington et al., 2013).

Wastewater treatment plants have been identified as hotspots of propagation and transfer of antibiotic resistance genes (ARGs) between bacteria as well as dissemination points of antibiotic resistant bacteria (ARBs) and ARGs to the environment (Droge et al. 2000; Schluter et al. 2007). The nutrient-rich, microbial-dense processes at a WWTP, in concert with the presence of antibiotic or other selection factors, has been shown to result in the persistence or enhancement of ARG and ARG concentrations in activated sludge (Stalder et al. (2013), wastewater effluent (Zhang, 2009); Ferreira da Silva (2006); Luczkiewicz, et al. (2010) and pertinent to this research, land-applied biosolids (Munir et al. (2011a), Munir et al. (2011b)).

The United States Environmental Protection Agency (USEPA) has long recognized the inherent biological risk associated with beneficial use of biosolids. Part 503 regulations were enacted to protect human health while allowing for the beneficial recycling of nutrients and cost-effective disposal of biosolids critical for a sustainable practice of wastewater treatment. However, public confidence in and positive perception of land application of biosolids has been hampered by the lack of scientific studies investigating the reduction or removal efficiencies of the wide range of pathogens in sludge treatments that generate biosolids. Critical review of sludge stabilization

technologies has increased with an eye toward better characterization of pathogen loading, both in terms of pathogen identification and quantification.

While better characterization is warranted, studies targeting a better understanding of the inactivation or removal mechanisms of pathogen and ARGs in sludge treatment processes are also sorely needed. Recent work has shown that thermal processes (thermophilic digestion and pasteurization) may not be effective in the inactivation of some pathogens and pathogen indicators (Higgins et al., 2007).There is growing support for improved characterization of pathogen removal, expanding the focus beyond the current treatment efficacy indicators (*Salmonella* and fecal coliforms) to determine inactivation rates for other pathogens (*Legionella pneumophila*, *Staphylococcus aureus*, *Campylobacter*, and *Clostridium difficile*, Viau et at., 2011), pathogen indicators (male coliphage, Viau et al., 2009), and pathogens containing antibiotic resistance genes (methicillin-resistant *Staphylococcus aureus*, MRSA, Viau et al., 2011; Lewis et al., 2002).

USEPA (2003) listed pathogenic Escherichia coli as one of six bacterial principal pathogens of concern that may be present in raw sludge with disease-causing potential. In addition, E. coli are Gram negative and comprise a large fraction of the fecal coliform group, which is used as a performance monitoring standard for Class A biosolids. Pertinent to thermophilic treatments, recent studies have found that E. coli are more thermotolerant than other bacteria measured as fecal coliforms (Spinks et al., 2006; Viau et al., 2011), resulting in differential survival of E. coli relative to other fecal coliforms and subsequent enrichment of E. coli in biosolids (Ziemba and Peccia, 2012). In addition, *E. coli* are common carriers of ARGs, with reports up to 40% of culturable *E. coli* in wastewater being resistant to at least one antibiotic (Jury et al., 2011; Garcia et al., 2007; Reinthaler et al., 2003).

S. aureus is a Gram positive pathogen of clinical significance. The presence of *S. aureus* in biosolids is variable, with early studies reporting no detections in aerobic compost (Burtscher et al., 2003) and anaerobically-digested biosolids (Burtscher et al., 2003; Rusin et al., 2003). However, Viau et al. (2009) reported the first detection of *S. aureus* via QPCR (nuc gene) in mesophilic and thermophilic anaerobic digester effluents and Borjesson et al. (2009) reported the mecA gene in digester centrate. The inconsistency in detection of *S. aureus* in biosolids relative

to raw influent suggests that sludge treatments either reduce the prevalence of *S. aureus* or receive highly variable levels of *S. aureus* in primary and secondary sludge, resulting in sporadic detections. MRSA specifically has been detected via culturing at wastewater treatment plant inlets, wet stream treatment processes, and the outlet (Borjesson et al., 2009; Borjesson et al., 2010; Thompson et al., 2012; Goldstein et al., 2012).

Few studies have investigated the fate of ARGs in sludge stabilization processes, such as mesophilic anaerobic digestion, thermophilic anaerobic digestion, and pasteurization of wastewater solids (Ghosh et al. (2009), Diehl and LaPara (2010), Ma et al. (2011), and Miller et al. (2013)). While all genes studied to date have shown some reduction during thermophilic treatment, results for tetracycline genes in mesophilic digestion have varied. This suggests that the digester microbial community and the operating conditions that influence the development and maintenance of that community play an important role in determining ARG fate. Aside from temperature, digester operating conditions and raw sludge characteristics (such as ARG composition or antibiotic and Ag NPs concentration) that might affect digester ARG composition have not been investigated.

Wastewater treatment plants serve a critical role in protection of human health and the environment through managing the quality of treated effluent and biosolids that are discharged to the environment. Moving sludge stabilization technologies into the future requires expansion of pathogen inactivation studies to assess treatment requirements for a broader range of pathogens and biological elements, such as antibiotic resistance genes, and addressing analytical limitations of assessing viability.

Research Questions

Research performed and reported herein sought to answer the following questions.

Question 1: What are the pathogen inactivation kinetics and mechanisms associated with E. coli, MRSA, and the ARGs housed within these pathogens in sludge stabilization technologies, including land application, mesophilic digestion, thermophilic digestion, and pasteurization? How is inactivation best assessed analytically?

Land application of biosolids is a potential route of dissemination for pathogens and ARGs to the environment. The sludge stabilization processes upstream of land application are critical points of control for these biological contaminants. However, assessing viability of a bacterial cell can be difficult with issues such as viable but nonculturable, DNA persistence, and repair of cell membrane integrity.

Question 2: Which environmental or operating factors influence ARG fate in anaerobic digestion?

The few studies of ARG fate during digestion have shown that different genes have differing levels of reduction, or even increase, during anaerobic digestion. Specifically, tetracycline genes often increase or remain unchanged in mesophilic digestion, while the same genes typically decrease in thermophilic digestion. Potential factors affecting ARG fate in digestion may include digester operating conditions (temperature), digester microbial community and ARG content, raw sludge microbial community and ARG content, and sludge contaminants, such as antibiotics and antimicrobials including nanosilver.

Question 3: Do environmental stresses (cold shock) instigate horizontal gene transfer during biosolids storage?

It was observed that concentrations of some ARGs (intI1 and sul1) increased during cold (4 °C) storage of raw sludge, while other ARGs (tet(O) and tet(W) were variable. These observations were extrapolated to consider the implications of cold weather storage on ARG content of biosolids, as often required in northern climates until a time suitable for land application.

Annotated Dissertation Online

Chapter 1: Introduction. The introduction chapter provides background, defines the focus of the research by outlining major research questions, and briefly summarizes each chapter.

Chapter 2: Inactivation of MRSA and Escherichia coli in various sludge treatments: Implications for pathogen and ARG loading in land application of biosolids. In this study, the inactivation kinetics of E. coli SMS-3-5 and a strain of community acquired MRSA were investigated under conditions representative of various sludge treatment technologies, including pasteurization,

thermophilic digestion, mesophilic digestion, and land application, to give insight into the mechanisms and extent of inactivation of the pathogen and the ARGs housed within these pathogens during sludge treatment. Plate counts, QPCR, and live/dead staining results are presented and discussed in the context of cell viability, DNA persistence, and treatment efficacy with respect to pathogens and ARGs. Research Questions 1 and 2 are addressed by this chapter.

Chapter 3: Mechanisms of ARG Persistence and Attenuation During Anaerobic Digestion. In this study, two tetracycline-resistant microorganisms and the ARGs contained within those microorganisms were monitored during mesophilic and thermophilic digestion batch reactors to investigate the role of host survival and horizontal gene transfer within the digester community on ARG content. In addition, the ARG concentrations of influent raw sludge and digested sludge were monitored in lab-scale mesophilic and thermophilic digesters to evaluate the impact of influent raw sludge ARG composition on digester effluent ARG composition. This study concluded that HGT is important more so in mesophilic digestion than thermophilic digestion due to the diverse microbial population and broad host range. ARB survival and growth is an important factor in ARG fate in both digestion regimes. Research Question 2 is addressed in this chapter.

Chapter 4: Effect of Silver Nanoparticles and Antibiotics on Antibiotic Resistance Genes in Anaerobic Digestion. This manuscript describes the ARG response in lab-scale mesophilic and thermophilic digesters that were supplemented with nanosilver and antibiotic sulfamethoxazole. This work investigated the potential for an antibiotic (sulfamethoxazole) or microbial active nanomaterial (nanosilver) to select for ARBs or ARG transfer. In this study, no correlation was found between antibiotic or nanosilver concentration and ARG response in thermophilic digestion suggesting that selection of ARBs over non-resistant microbes is not a dominant ARG persistence mechanism. Thermophilic digestion reduced a wide variety of ARGs, whereas tetracycline genes were not reduced and sometime increased in mesophilic digestion. This work was presented at the 2010 WEF Biosolids and Residuals Conference in Raleigh, North Carolina in March 2010 and was published in Water Environment Research. Research Question 2 is addressed by this chapter.

Miller, J. H., Novak, J. T., Knocke, W. R.,; Young, K., Hong, Y., Vikesland, P. J., Pruden, A., 2013. Effect of Silver Nanoparticles and Antibiotics on Antibiotic Resistance Genes in Anaerobic Digestion, Water Environment Research, (85) 5: 411-421.

Chapter 5: ARG increase in response to cold temperature exposure: Implications for winter storage of sludge and biosolids. In this study, Class B mesophilic digested sludge was stored at 4 °C, 10 °C, and 20 °C for a period of 4 months. Each month, TS, VS, pH, alkalinity, and genes encoding antibiotic resistance to sulfonamides (*sul*1) and the integrase enzyme (*int*I1) of Class 1 integrons were measured to evaluate the impact of storage temperature on ARG fate. This study found increases in ARGs during biosolids storage and identifies changes in operational protocols that could help reduce ARG loading to the environment through land application of stored biosolids. Research Question 3 is addressed by this chapter.

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CHAPTER 2

Inactivation of MRSA and Escherichia coli in various sludge treatments: Implications for pathogen and ARG loading in land application of biosolids

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Key words: Biosolids, land application, mesophilic digestion, thermophilic digestion, pasteurization, MRSA, *Staphylococcus aureus*, *Escherichia coli*, antibiotic resistance genes

Introduction

Approximately 7.18 million tons of biosolids are generated per year, of which 3.95 million tons are applied to land (NEBRA, 2007). Sludge stabilization and land-applied biosolids represent one of the largest opportunities for energy generation, resource recovery, and cost-effective (even profit-producing) sustainability in this country, but also one of the greatest challenges to protection of human and environmental health. Land application of biosolids is receiving critical attention in response to reports of viable but nonculturable states of pathogens and pathogen indicators (Higgins et al., 2007), the need for more quantitative characterization of pathogen loads for microbial risk assessments (Viau et al., 2011), recent reports of adverse health impacts by people living close to land application sites (Lewis et al., 2002), and identification of wastewater treatment plants as dissemination points for antibiotic resistance genes to the environment (Droge et al., 2000; Schluter et al., 2007). All of these issues stem from the need for greater understanding of pathogen inactivation kinetics and mechanisms during Class A and Class B sludge stabilization processes preceding land application of biosolids.

While the reuse of biosolids has many benefits, public confidence in and positive perception of land applied biosolids has been hampered by the lack of scientific studies investigating the reduction or removal efficiencies of the wide range of pathogens in sludge treatments that generate biosolids. There is growing recognition of the need for improved characterization of pathogen removal, expanding the focus beyond the current treatment efficacy indicators (*Salmonella* and fecal coliforms) to determine inactivation rates for other pathogens (e.g., *Legionella pneumophila*, *Staphylococcus aureus*, *Campylobacter*, and *Clostridium difficile*, Viau

et at., 2011), pathogen indicators (male coliphage, Viau et al., 2009), and pathogens containing antibiotic resistance genes (methicillin-resistant *Staphylococcus aureus* (MRSA), Viau et al., 2011; Lewis et al., 2002).

The United States Environmental Protection Agency (USEPA) Part 503 pathogen reduction and land application rules are based on operational guidelines intended to reduce microbial risk through appropriate treatment of solids prior to land application, limiting exposure through site restrictions, and demonstration of pathogen reductions through monitoring (NRC, 2002). Part 503 time-temperature requirements were based on monitoring results from full-scale plants with respect to *Ascaris* and enterovirus with particular emphasis on *Salmonella*. Part 503 rules established monitoring requirements based on *Salmonella* or fecal coliform densities as representative of the adequate reduction of all pathogens (USEPA, 2003). More recent studies investigating the occurrence of other pathogens has revealed that fecal coliform monitoring is not the best indicator of reductions for all pathogens (Viau et al., 2009). In addition, prescribed technologies do not necessarily result in appropriate reductions for all pathogens, and more importantly, Class A biosolids are not necessarily pathogen-free (Viau et al., 2011). Class B biosolids are expected to retain some pathogens and as such, land application rules restrict site use to limit exposure. However, it is difficult to assess the efficacy of these restrictions without a clear understanding of the extent and magnitude of pathogen loading.

In order to advance sludge stabilization technologies in a way that minimizes potential risk to public health, pathogen inactivation mechanisms require a more detailed understanding. This will enable evaluation of treatment requirements for a broader range of pathogens and biological elements, such as antibiotic resistance genes, while also addressing analytical limitations of assessing viability. Indeed, the definition of bacterial death is a debated topic. Should death be described as an inability to resuscitate, replicate, or conjugate, or does it require complete destruction of the cell? Is death characterized as the inactivation of the one critical cellular component that is required for cell function? What treatment conditions are required to make these things happen? And finally, how is efficient treatment assessed? Pathogen monitoring has been hampered by what is termed viable but nonculturable (VBNC) states of some bacteria when exposed to stress, such as heat stress in thermal treatments of sludge. Live/dead staining assesses only the membrane integrity of a cell, which may not be a true indicator of cell death. QPCR

technologies have been criticized as overly conservative because QPCR detects all DNA, including extracellular DNA and DNA contained in dead cells. New advances in pyrosequencing are attractive, but still suffer from limitations associated with primer specificity (the length of amplicon resulting from 16S rRNA primers required to achieve determination of genus level determinations) and still do not distinguish between contributions from live and dead bacteria.

The USEPA (2003) listed pathogenic *Escherichia coli* as one of six principal bacterial pathogens of concern that may be present in raw sludge with disease-causing potential. In addition, *E. coli* are Gram negative and comprise a large fraction of the fecal coliform group, which is used as a performance monitoring standard for Class A biosolids. Pertinent to thermophilic treatments, recent studies have found that *E. coli* are more thermotolerant than other bacteria measured as fecal coliforms (Spinks et al., 2006; Viau et al., 2011), resulting in differential survival of *E. coli* relative to other fecal coliforms and subsequent enrichment of *E. coli* in biosolids (Ziemba and Peccia, 2012). The underlying physiological reason behind *E. coli*'s persistence during thermophilic treatments has been reported to be entrance into a VBNC state (Higgins et al., 2007). The VBNC state has been described for many different bacteria and is characterized by the inability to grow in culture using standard culturing methods. VBNC bacteria can be resuscitated or reactivated and resume culturability and infectability under favorable conditions once the stress has been removed (Oliver, 2010). In addition, *E. coli* are common carriers of ARGs, with reports up to 40% of culturable *E. coli* in wastewater being resistant to at least one antibiotic (Jury et al., 2011; Garcia et al., 2007; Reinthaler et al., 2003).

MRSA is a Gram positive pathogen of serious clinical concern. MRSA has been detected via culturing at wastewater treatment plant inlets, wet stream treatment processes, and the outlet (Borjesson et al., 2009; Borjesson et al., 2010; Thompson et al., 2012; Goldstein et al., 2012), but the presence of *S. aureus* and more specifically, MRSA, in biosolids is still unclear. Burtscher et al., (2003) collected 46 samples from aerobic compost and anaerobically-digested biosolids, but did not detect viable *S. aureus* by culturing or PCR. Rusin et al. (2003) detected *S. aureus* via culturing in raw sewage, but not in finished biosolids arising from mesophilic anaerobic digestion, temperature phased anaerobic digestion, or composting. However, Viau et al., 2009 reported the first detection of *S. aureus* via QPCR in 19% (of 16 total) MAD samples and 50% (of 8 total) TPAD samples. *S. aureus* was not detected in composted mesophilic digested

biosolids (10 samples). Borjesson et al. (2009) detected high concentrations of the MRSA-associated methicillin resistance gene, *mecA,* in centrate in 4 out of 12 monthly samples, but did not monitor digested solids. It is not clear (Borjesson et al., 2009) whether the infrequent yet high concentrations of *mecA* in the digester effluent were caused by sporadic high influent concentrations or inconsistent reductions in the digester. Given the detection of *S. aureus* in biosolids (Viau et al., 2009) and the noted presence of *mecA* in centrate (Borjesson et al., 2009), it is plausible that MRSA could persist in finished biosolids. However, the inconsistency in detection of *S. aureus* in biosolids relative to raw influent in these cited studies suggests that sludge treatments either reduce the prevalence of *S. aureus* or receive highly variable levels of *S. aureus* in primary and secondary sludge resulting in sporadic detections.

In this study, the inactivation kinetics of *E. coli* SMS-3-5 and a strain of community acquired MRSA were investigated under conditions representative of various sludge treatment technologies to give insight into the mechanisms and extent of inactivation of the pathogen and the ARGs housed within these pathogens. *E. coli* were included in this study because of the potential for pathogenicity (USEPA, 2003) and antibiotic resistance (Jury et al., 2011), *E. coli* is Gram negative, and *E. coli* comprise a recalcitrant portion of the fecal coliform group (Ziemba and Peccia, 2012) on which Class A and Class B land applied biosolid regulations are based. MRSA was included in this study because it is Gram positive, clinically relevant, and an emerging concern in biosolids. Pure cultures of E. coli SMS-3-5 and MRSA were spiked into batch reactors and incubated at temperatures representing pasteurization (70 °C), thermophilic digestion (53 °C), mesophilic digestion (37 °C), and land application (20 °C). Plate counts, QPCR, and live/dead staining results are presented and discussed in the context of cell viability, DNA persistence, and treatment efficacy with respect to pathogens and ARGs.

Materials and Methods

Bacterial strains. *E. coli* SMS-3-5 (ATCC# BAA-1743, phylogenetic group D, serotype O19:H34) is a Gram negative, multi-drug resistant, nonvirulent strain originally isolated from an industrial, toxic metal-contaminated coastal environment (Stepanauskas and Lindell, 2005). This strain is resistant to β-lactam, aminoglycoside, quinolone, macrolide, sulfonamide, and tetracycline antibiotics, copper and zinc metals, quaternary ammonium disinfectants with a

confirmed presence of *tet*(A) and *sul2*, which reside on the pSMS35_130 plasmid (Fricke et al., 2008).

Methicillin-resistant *S. aureus* (MRSA, ATCC# BAA-1556) is a Gram positive, USA300 clone of community-acquired MRSA (Diep et al., 2006; Diep et al.,2008) with confirmed presence of *mecA* on type IV staphylococcal chromosomal cassette (SCCmec IV) mobile genetic element. This strain is also resistant to β-lactams and ciprofloxacin (located on the chromosome) and tetracycline via confirmed presence of *tet*(K), which resides on a plasmid. Plasmid ARGs also encode resistance to macrolides (erythromycin), lincosamides (clindamycin), streptogramin B, and mupirocin.

Agar Plate Preparation. *E. coli* in pure culture were grown on LB broth agar (35 g/L in 100mmx15mm Petri dishes). MRSA in pure culture were grown on brain heart infusion agar (BHI, 49 g/L in 100mmx15mm Petri dishes).

E. coli in sludge were enumerated using Standard Method 9222G (APHA, 1995). Briefly, mFC agar was added to pre-autoclaved reverse osmosis water, dissolved by gently heating, and cooled in a 55 °C water bath. Rosolic acid was added for a final concentration of 1%. Agar was mixed at low speed on a stir plate prior to pouring in 60mmx15mm Petri dishes. Plates were stored at 4 °C prior to use.

MRSA in sludge was isolated using Muehller Hinton agar augmented with ciprofloxacin (2 mg/L), oxacillin (32 mg/L), and cycloheximide (200 mg/L). Specifically, Mueller Hinton agar (MH, final concentration 38 g/L) was added to reverse osmosis water, dissolved by heating, and autoclaved at 121 °C for 20 minutes. The agar was cooled in a 55 °C water bath prior to addition of filter-sterilized solutions of antibiotics and antifungal Agar was mixed at low speed on a stir plate prior to pouring 100mmx15mm petri dishes.

Cell Culturing/Plating.

MRSA. Plating of cells was done immediately after sampling by serial 10x dilutions of the original sample in autoclaved phosphate-buffered saline (PBS) solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.76 nM KH2PO4, pH = 7.4) and inoculating two antibiotic-amended plates with 100 µl of diluted sample. Prior to plating, culture tubes were vortexed at a moderately

high speed for 30 seconds to dislodge bacteria from sludge particles. Inoculated plates were incubated at 37 °C for 3 days prior to cell count. The use of two antibiotics (ciprofloxacin and oxacillin) precluded the growth of any other bacteria in sludge in all experiments as shown by no growth on plates with unamended "blank" sludge. All plates were incubated at 37 °C for 3 days to allow sufficiently sized colonies for counting. Colony counts ranging between 25 and 250 CFU were targeted. On occasion, two dilutions would result with colonies in this range. Plate counts for all valid dilutions were recorded.

E. coli. *E. coli* was enumerated using Standard Method 9222G (APHA, 1995). During sample analysis, sludge samples (100 ul) were 10x serially diluted in autoclaved PBS. A 500ml aliquot of each dilution or the undiluted 1x original sample was further diluted into 4.5ml PBS and vortexed a moderately high speed for 30 seconds to dislodge bacteria from sludge particles. The entire contents of the secondary dilution (i.e., 5 ml containing 500ml diluted sample) were filtered under sterile conditions on 47mm diameter, 0.45 um mixed cellulose ester filters with grid lines (Millipore® S-pak membrane filter HAWG047S6). The filter was placed on mFC agar plates and incubated at 44 °C for 24 hours. After initial incubation, the plate image was captured on a Gel Doc lab imager. The filter was then transferred to ECMUG agar plates and incubated further at 44 °C for 24 hours to positively identify *E. coli* colonies with fluorescent halos. Plates with colony counts ranging between 20 and 80 CFU were targeted. At high concentrations, *E. coli* was the only microorganism to grow in the diluted samples because other background bacteria were diluted out.

Pure Culture Stock Preparation. Pure culture stocks of bacteria were grown by spiking six 100 ml flasks of broth (LB for *E. coli* and BHI for MRSA) with 1ml of freezer stock (-80 °C) each and grown to late log phase. Cells were harvested by centrifugation at 10000 rpm for 10 minutes, rinsed in phosphate buffered saline, and re-concentrated. The rinse step was repeated three times prior to final resuspension in PBS (30 ml total). The absorbance at 600nm was measured for a 10x dilution of the concentrated cell suspension. An absorbance of 1.2 approximated a cell density of 10^8 cells/ml (10^9 cells/ml in the undiluted suspension).

Experimental Setup. Digested sludge (4.5ml) was aliquoted in culture tubes (100x17mm, Fisher Scientific) and placed in a water bath at the test temperature (i.e., 20 °C, 37 °C, 53 °C, or 70 °C).

Three replicates were unamended and served as a control blank. Other replicates received a spike of the concentrated pure culture of pathogen. Over a range of time points ranging from a few minutes (at 70 °C) to a few weeks that was suitable for capturing the death/decay curve, triplicate sample tubes were removed and immersed in a water/ice bath to quench the temperature reaction. After quenching, an aliquot of the sample was stored at -20 °C until DNA extraction while plating was done immediately.

Quantification of ARGs. DNA was extracted from 175 µL of sludge samples using the MagMax Total *Nuc*leic Acid Extraction Kit (Ambion, Life Technologies) according to manufacturer's protocol. Extracted DNA was diluted 50× to minimize inhibitory effects as determined by analyzing a dilution series of DNA extract. Diluted DNA extracts were stored at -20°C until analysis by QPCR for bacterial 16S rRNA genes, *tet*(G), and *tet*(W). A 10 µL reaction mixture was comprised of 5.0 µL SsoFast Evagreen Supermix (Bio-Rad, Hercules, California), 0.8 µL of each 5 µM primer (Table 1), 2.4 µl molecular biology grade water, and 1 µL of DNA template. All samples were quantified in triplicate. Standards prepared from serial dilutions of cloned genes ranged from 10^1 to 10^7 gene copies per µL. Standards and a reagent blank were included in each run. ARG gene copy numbers as plotted are a result of adjustment by three multiplication factors, including factors related to extraction of DNA from the sample (typically 0.3x), dilution of the extract to reduce QPCR inhibitor concentrations (1x in PBS and 100x in sludge), and unit conversion from ul to ml (1000x). [The exception to these values was the 60-day sample for MRSA at 53 °C in digested sludge, which used a 2.0x extraction factor and 1x inhibitor dilution factor.] Based on these values, the lowest standard on the QPCR curve (LOQ, 10 gcn per ul of extract) would translate to 3.0 x 10^3 gcn/ml PBS or 3.0x10^5 gcn/ml digested sludge. ARG copy numbers in digested sludge samples are further adjusted by the total solids of the sample, which ranged from 8.5 to 14.0 g TS/L, yielding units of gcn/g TS and an LOQ on the order of 10^7 gcn/g TS. An experimental replicate yielding a non-amplified threshold cycle in at least two analytical triplicates were considered non-detect. Experimental replicates yielding detections in at least two analytical triplicates were averaged, neglecting the single non-detect. Time point averages where at least two experimental triplicates were non-detect are reported as non-detect, otherwise time point averages where at least two experimental triplicates are detected are reported as an average

of the two detections, neglecting the non-detected replicate. Non-detections are noted on the figures.

Table 2-1. Primer sequences and annealing temperatures for QPCR assays.

Gene	Primer sequence	Amplicon length (bp)	T_a (°C)	Reference
*gad*A/B	(F) 5'-ACC TGC GTT GCG TAA ATA (R) 5'-GGG CGG GAG AAG TTG ATG	669	69.0	McDaniels et al., 1996
tet(A)	(F) 5′-GTA ATT CTG AGC ACT GTC GC (R) 5′-CAT AGA TCG CCG TGA AGA GG	1054	57.2	McKinney et al., 2012
sul2	(F) 5'-TCCGGTGGAGGCCGGTATCTGG (R) 5'-CGGGAATGCCATCTGCCTTGAG	191	67.2	Ma et al., 2011
nuc	(F) 5'-CTTTAGCCAAGCCTTGACGAACT (R) 5'-CCTGAAGCAAGTGCATTTACGA	166	60.0	Graber et al., 2007
mecA	(F) 5′-CGCAACGTTCAATTTAATTTTG TTAA (R) 5′-CCACTTCATATCTTGTAACG	1018	51.7	McKinney et al., 2012
tet(G)	(F) 5'-GCAGAGCAGGTCGCTGG (R) 5'-CCYGCAAGAGAAGCCAGAAG	134	64.2	Ma et al., 2011

T_a, Annealing Temperature

pH and TS/VS. Total and volatile solids (Method 2540-G) and pH (Method 4500) were analyzed at the start and end of the studies as specified in Standard Methods for the Examination of Water and Wastewater (APHA, 1995).

DNase 1 treatment. MRSA and *E. coli* sludge 60-day samples from the long term 53 °C experiment were treated with DNase 1 enzyme to digest extracellular DNA within the sample (Dominiak et al., 2011). DNase 1 (150 U) was added to 25 ul sludge sample, vortexed, and incubated at 37 °C for 30 minutes. After incubation, EDTA was added to a final concentration of 30 mM and incubated at 65 °C for 10 minutes to stop the DNase reaction. Following the stop reaction, DNA was extracted as described above.

LIVE/DEAD® BacLight™ bacterial viability staining. The LIVE/DEAD® BacLight™ bacterial viability staining kit (L7007, Life Technologies) uses Syto 9 green fluorescent and propidium iodide red fluorescent *nuc*leic acid stains to stain cells without compromised membranes (live, viable) and cells with compromised membranes (dead), respectively. For PBS samples, Component A (10 ul, 1.67 mM Syto 9, 1.67 mM propidium iodide) and Component B

(10 ul, 1.67 mM Syto 9, 18.3 mM propidium iodide) were diluted (200x) in sterile PBS for a total volume of 2 ml. For sludge samples, the stain proportions were adjusted (7.5ul Component A, 2.5 ul Component B) and diluted (100x) to 1ml total volume in sterile PBS to allow a higher concentration and longer viewing time of Syto 9 fluorescence. In PBS, samples (500 ul) were stained with the dye mixture (120 ul) for 15 minutes while stored in the dark. After incubation, 500 ul of the stained sample was diluted in 4.5 ml sterile PBS, vortexed to separate cells, and aseptically filtered on black polycarbonate membranes (Whatman *Nuc*leopore, 22mm diameter, 0.2 um pore) using a 25mm glass filter holder with fritted glass support (VWR # 26316-690), 15ml glass funnel (VWR # 26316-694), aluminum clamp (VWR # 26316-700), silicone stopper #5 (VWR# 26316-702) and a filtering flask. Membranes were mounted with mounting oil on a glass slide and covered with cover slip. For sludge samples, 50 ul of sludge sample was stained with 12 ul of dye mixture, incubated for 15 minutes in the dark, then direct pipetted (10 µl) on glass slide. The sludge matrix was able to "quench" the excess dye without diluting and filtering as with the PBS samples. Moreover, filtering sludge samples concentrated the fluorescence of the background sludge on the filter surface making it more difficult to see individual cells. The stained sludge was covered with a cover slip, then sealed with fast drying, clear nail polish. All slides were stored at -20 °C until analysis within 3 days. After this length of time, cell counts deteriorated as a result of the dyes fading (data not shown).

The slides were viewed on a fluorescent microscope using filter sets that contained 480 ± 20nm excitation filter, a 505-nm long-pass dichroic mirror, and a 535 ± 25 nm emission filter (green dye) and a 545 ± 15 nm excitation filter, a 570-nm long-pass dichroic mirror, and a 610 ± 37 nm emission filter (red dye). Approximately 20 images (field of view) per slide were obtained with Axiovision software. Cell counts and fluorescence area were automatically counted for all images within one experiment by optimizing background subtraction, threshold, and pixel size parameters in a macro developed within ImageJ (Rasband, 2012). Viability was calculated as a percent of live (green) cell counts to the total of separate live and dead (red) cell counts. [Although viability as calculated by total area was similar in magnitude and time trends to viability as calculated by cell counts, viability based on cell count was selected because the cell area associated with the rod-shaped *E. coli* varied depending on orientation of the cell on the slide. MRSA cells are spherical and were not affected by orientation.] The interference from the

background fluorescence of the combined red and green images did not allow accurate automated cell counting, so it was not possible to account for cells that stained with both green and red dyes. As such, the live percentages are likely overestimates of actual viability; however, the trend of viability is considered unaffected assuming the proportion of cells per field of view that stained with both dyes remained constant. The experiments with *E. coli* in digested sludge at 37 °C and 53 °C had shown that the target pathogen was a small part of the overall community when spiked into sludge and the viability percentage of sludge samples remained constant throughout all sludge experiments (data not shown). LIVE/DEAD® staining of sludge samples was not continued for MRSA experiments.

Results

MRSA

Land application simulation (20 °C)

MRSA plate counts declined steadily throughout the study in phosphate buffered saline (PBS) from 2.4 x 10^9 CFU/ml starting concentration to 7.5 x 10^5 CFU/ml on Day 60 (Figure 1). It is likely that some replacement growth was maintained in the PBS solution by live cells "feeding" on dead cell components. The percentage of viable cells as measured with live/dead staining is also a declining trend. MRSA DNA concentrations (*nuc* and *mecA*) remained consistent throughout the PBS study despite the declining plate counts, indicating DNA persistence within dead, yet unlysed cells or as extracellular DNA.

In digested sludge (Figure 2), MRSA plate counts initially increased through the first day, then quickly deteriorated to no growth (no colonies grown when 100 µl undiluted sludge was plated) by Day 20. MRSA DNA also declined, albeit at a slightly reduced rate relative to plate counts, reaching concentrations close to the limit of quantification (LOQ) (*nuc*) or non-detect (*mec*A) by Day 20 and reaching non-detect for both genes by Day 60. In contrast to *mec*A and *nuc*, which are housed within the spiked pathogen, *tet*(G) was measured as representative of the background bacterial community. The concentration of *tet*(G) remained at consistent levels throughout the study.

Mesophilic Digestion (37 °C)

In PBS (Figure 3), MRSA plate counts and DNA remained consistent throughout the study, showing the persistence of the bacterial cells. The percentage of viable cells decreased up to Day 10, then increased and remained constant to Day 40. In contrast, in digested sludge (Figure 4), MRSA plate counts rapidly declined with the first day of incubation, leveled off for a few days, then went to no plate growth sometime between Day 5 and Day 10. As in the 20 °C experiment, the degradation of the DNA lagged behind CFU, where *mec*A and *nuc* were both detected just above the LOQ on Day 10, but then both genes reached non-detect by Day 20. *tet*(G) remained at consistent levels throughout the study, indicating the background ARG content was not affected.

Thermophilic Digestion (53 °C)

In PBS (Figure 5), MRSA plate counts declined quickly from 4.8 x 10^6 CFU/ml to nondetect within one hour. *mec*A declined by approximately 2-log shortly after the first hour, but then remained at a 5-log concentration through the end of the 2.5 days. The *nuc* gene did not decline during the study. The live percent of total cells were variable throughout the study. In digested sludge (Figure 6), MRSA plate counts also decreased to nondetect within the first hour. MRSA DNA did not decline at all within the time of the experiment (2.5 days), mirroring the *tet*(G) concentrations in the background sludge.

The experiment at 53 °C in sludge was repeated and carried out for a longer period of time (60 days, Figure 7). Again, the plate counts declined to nondetect within the first hour (data not shown), but surprisingly the MRSA DNA persisted with only a modest decline throughout the entire 60-day time period. The trend in the background community gene, *tet*(G), mirrored the trend in the MRSA DNA concentrations. Sludge samples at 60 days were treated with DNase 1 to remove extracellular DNA. DNA was extracted and analyzed from triplicate samples with and without DNase treatment. While DNase treated samples were lower in *mec*A and *nuc* concentrations, the difference was not significant and a similar trend was found in *tet*(G) (Figure 8). In addition, DNase treated and untreated samples were viewed with live/dead staining and background fluorescence of the DNase treated samples was reduced. These data suggest- that the *mecA* and *nuc* genes amplified by QPCR were contained within live or unlysed dead MRSA cells and were not present as extracellular material.

Pasteurization (70 °C)

The spiked culture tubes (PBS or digested sludge) were placed in a water bath at 70 °C to preheat. The caps were closed during preheating, but were removed to add the pathogen. Samples were spiked, incubated for the set time, and immediately placed in an ice bath to quench the reaction. The interim short incubation times (first time point was 20 seconds) likely introduced large error into the resultant plate counts for time points less than one minute. After one minute, all plate counts were nondetect both in PBS (Figure 9) and sludge (Figure 10). In PBS and sludge, *mecA*, *nuc*, and *tet*(G) concentration and viable percentage remained consistent throughout the 30-minute study. The experiment length was 30 minutes to meet the USEPA's designation of 70 °C for 30 minutes for pasteurization.

E. coli SMS-3-5

Mesophilic Digestion (37 °C)

Studies conducted with *E. coli* SMS-3-5 at mesophilic temperature in PBS did not show a substantial decline in plate counts or *gad*A/B and *tet*(A) genes (Figure 11). Bacteria can likely continue to survive in PBS without external nutrients because of the nutrition provided by dying cells. Re-appropriation of dead cells may be the reason behind the rise in the relative proportion of viable cells to dead cells (% live) as measured by membrane integrity dyes. *E. coli* SMS-3-5 plate counts in digested sludge (Figure 12) showed a slightly more rapid decline than MRSA, going to non-detect by Day 7. However, *E. coli* SMS-3-5 ARGs(*tet*(A) and *sul*2) and microbe-specific gene (*gad*A/B) remained at a high concentration throughout the 20-day experiment.

Thermophilic Digestion (53 °C)

Similar studies conducted with *E. coli* SMS-3-5 at 53 °C in PBS (Figure 13) and thermophilic digested sludge (Figure 14) had similar results to MRSA. In PBS, plate counts decreased to non-detect levels within 5 days. *E. coli* genes (*gad*A/B and *tet*(A)) declined approximately 2 log in a similar time frame, but then remained consistent through 24 hours. Viability percentages based on membrane integrity are variable, likely as a result of the elevated background fluorescence in later time points that hampered automated cell counting. The cell shapes as viewed in these samples relative to the mesophilic samples were less distinct perhaps as a result of DNA from

lysed cells clouding the overall fluorescence or damage to the cells themselves. In digested sludge, plate counts showed a similar decline to non-detect levels within 1 day, but a 2 log decline in gene concentrations was less abrupt and took place over the entire 53 hour time period.

As with MRSA, the experiment with *E. coli* SMS-3-5 in digested sludge was repeated and extended to 60 days to attempt to capture the decay of DNA commensurate with plate counts. As in the first study, plate counts were below detection within the first day (data not shown). Between initial and Day 20 time points, *tet*(G) concentration remained consistent at 10^8 gcn/ml, whereas a 1.5 to 2 log decrease in *gad*A/B, *tet*(A), and *sul2* occurred. There was a larger divergence of *E. coli* genes from the background sludge gene, *tet*(G), between Day 20 and Day 60. A portion of ARG decline can likely be attributed to poor DNA extraction efficiency in Day 60 samples relative to earlier samples, but a comparison of *E. coli* genes (*gad*A/B, *tet*(A), and *sul2*) to background gene, *tet*(G), also suggests that E. coli DNA is degrading faster relative to the background biomass. *tet*(G) concentration was reduced by 2-log relative to the 4-log reduction in *E. coli* ARGs between Day 20 and Day 60.

Discussion

A comparison of the 20 °C and 37 °C experiments with MRSA in digested sludge to the experiments in PBS show an enhanced degradation of colony plate counts and DNA in digested sludge relative to PBS. This contrast highlights not only the importance of biological and abiotic conditions of the matrix on degradation kinetics, but also suggests that MRSA cell death in sludge is not caused by temperature alone. Other factors (pH, protozoa concentration) have been shown to affect survival of pathogens in sludge (Wichuk et al., 2007; USEPA, 2003), thus it is likely that the conditions of the sludge environment were ill-suited for MRSA's survival. However, the lag in degradation of DNA behind plate count decline in sludge implies that predation may not be a large contributor either. If the pathogen were consumed by a predator, it seems likely that the both cell count and DNA would disappear simultaneously at the time of engulfment. A lag in DNA degradation behind plate count decline could be explained by protection of extracellular DNA against nuclease attack by binding with humic acids and other forms of organic matter (Mao et al., 2014; Pietramellara et al., 2009; Levy-Booth et al., 2007),

both of which are plentiful in sludge environments. Regardless, both MRSA plate counts and DNA, including ARGs, perished during long-term operations (greater than 20 days) at 20 °C and 37 °C. These are positive results considering current time-temperature requirements for 37 °C treatments is 20 days (USEPA, 2003).

MRSA DNA data also suggest that no horizontal gene transfer occurred between the introduced pathogens and the sludge community because *mecA*, which is located on the chromosomal SCC mobile genetic element, had a similar degradation pattern as the pathogen chromosomal DNA, which is not prone to genetic transfer. Normalizing pathogen ARG (*mecA*) to pathogen DNA (*nuc*) shows a consistent ratio, supporting the inference of no horizontal gene transfer taking place (Merlin et al., 2011). In addition, the degradation of the pathogen ARG (*mecA*) relative to the consistent concentrations of ARG *tet*(G) measured in the background sludge community shows that different processes are at work on the pathogen ARG and community as a whole.

E. coli SMS-3-5 data at 20 °C in PBS (Mazzochette, 2013) showed a slight degradation of plate counts (1 log over 50 days) with similar degradation of *gad*A/B DNA. In sludge at 20 °C (Mazzochette, 2013), there was a 5-log degradation of *E. coli* plate counts over 40 days with a corresponding 3-log reduction in *gad*A/B DNA with the greatest rate of degradation occurring in the first 30 days followed by very little continued reduction. It appears from these degradation trends of plate counts and DNA that *E. coli* could persist for quite some time at low levels in land application conditions, whereas MRSA plate counts and DNA were non-detect by 20 days. Also in contrast to MRSA, there was no apparent lag in *E. coli* DNA degradation behind plate count decline. This could indicate preferential predation by protozoa on *E. coli* relative to MRSA. Alternatively and probably more likely, it is possible that the Gram positive cell wall of a dead MRSA cell requires more time to break down and thereby retains a protective structure around intracellular DNA allowing it to resist degradation longer than DNA in Gram negative *E. coli* cells. However, this was not the case with *E. coli* SMS-3-5 at 37 °C in digested sludge, where plate counts reached background within 7 days while DNA persisted for the length of the experiment (20 days, Figure 12).

At 53 °C and 70 °C, MRSA plate counts were non-detect within 1 hour and 1 minute, respectively, in both PBS and sludge. At 53 °C, *E. coli* plate counts also reached non-detect

quickly (32 minutes in sludge and by 4 hours in PBS). The high temperature obviously plays a key role in loss of culturability regardless of the matrix. These experiments were originally carried out to the USEPA-required time-temperature length (49 hours at 53 °C and 30 minutes at 70 °C); but it became apparent that this time frame was not adequate to capture possible degradation of DNA as observed at lower treatment temperatures.

As such, the experiment at 53 °C was repeated for MRSA and *E. coli* and extended to 60 days. MRSA and *E. coli* plate counts at 53 °C in sludge declined as in previous experiment, but interpretation of DNA data from the extended thermophilic digestion (53 °C) and pasteurization (70 °C) experiments is less clear, likely because of the potential for some pathogens to enter a VBNC state at high temperatures. The conditions and requirements for bacteria to enter a VBNC state, as well as the conditions and requirements for bacteria to resuscitate, are debated in literature. However, pertinent to high temperature sludge treatments, numerous studies have noted the decline in fecal coliforms or *E. coli* plate counts following thermophilic digestion followed by a subsequent increase in plate counts following centrifuge dewatering (Qi et al., 2004; Iranpour et al., 2003; Monteleone et al., 2004; Higgins et al., 2007). Higgins et al. (2007) attributed the 3 to 4-log increase in *E. coli* and fecal coliform densities following centrifuge dewatering of thermophilic digested sludge to a resuscitation of bacteria from a VBNC state, concluding that the increase was too large to be accounted for by regrowth in such a short period of time (~20 minute centrifuge retention time). There is documentation of MRSA entering a VBNC state in response to antibiotics (Pasquaroli et al., 2013) or low temperature (Masmoudi et al., 2010), but only a few studies have documented tentative observations commensurate with MRSA entering a VBNC state in wastewater conditions (Borjesson et al., 2010; Ohlsen et al., 2003). However, Oliver (2010) notes that the list of bacteria capable of entering the VBNC state is always growing, adding to the inclusion of such pathogens as *E. coli*, *Salmonella*, *Shigella*, and *Pseudomonas aeruginosa*. It is not unrealistic to suggest that *S. aureus* or MRSA could enter a VBNC state in response to high temperature sludge treatments.

In a VBNC state, the pathogens would not be detectable by traditional culturing methods. Moreover, live/dead staining and QPCR, which are indicators of uncompromised cellular components, may not give an accurate picture of cell viability. For example with the live/dead stain, cell membranes are permeated by Syto 9 (green fluorescence) nucleic acid stain, but

uncompromised cell membranes do not permit the intrusion of propidium iodide (red fluorescence). Compromised cell membranes will be permeated by both dyes, but propidium iodide fluorescence will reduce Syto 9 fluorescence such that it is considered that red cells have a compromised cell membrane and green cells do not. However, there is ample evidence to suggest that a compromised cell membrane is not an indicator of cell death, but rather a potentially transient injury that cells can repair during resuscitation (Mackey et al., 1991 and 1993; Lee and Kaletunc, 2002; Villarino et al., 2000; Berney et al., 2007; Guernec et al., 2013). As such, live/dead staining (of the PBS experiments samples) may not be conclusive because there is evidence that VBNC cells may have cell membrane damage allowing dye penetration, but would be able to be repair the membrane and resuscitate given the appropriate environmental conditions, i.e., the live/dead assay may show cell membrane damage, but is not an adequate indicator of cell death. Alternately, QPCR amplifies persistent DNA in samples, including extracellular DNA as well as DNA housed in live, VBNC, and dead but unlysed cells. Numerous studies have shown the resistance of intracellular and extracellular DNA to thermal degradation (Yap et al., 2013; Masters et al., 1998) and persistence of extracellular DNA in various environments for long periods of time ((Mao et al., 2014; Pietramellara et al., 2009; Levy-Booth et al., 2007). As such, QPCR even when coupled with viability dyes that permeate compromised membranes may not be a good indicator of viable pathogen presence under conditions that induce the VBNC state by cell membrane damage.

Given the individual limitations of each viability assay (QPCR, live/dead, culturing), it seems likely that a combination of assays may be required to assess the treatment efficiencies of pathogens at high temperatures that could induce VBNC states. For these experiments at 53 °C in sludge, the persistence of DNA beyond the time required for DNA degradation in sludge at 20 °C or 37 °C suggests that there is a differential persistence of DNA or the pathogen cell housing the DNA either through 1) entering the VBNC state or 2) unlysed dead cells affording some amount of protection to DNA housed within or 3) abiotic or biological differences resulting in the survival of extracellular DNA in thermophilic digested sludge relative to mesophilic sludge.

Considering these options individually for each pathogen, it is helpful to first consider the QPCR-amplified DNA trends of *E. coli* in sludge. At 53 °C in sludge, despite a rapid (<30 minutes) plate count drop to non-detect, *E. coli* DNA followed a slow degradation trend over

time. The *E. coli* DNA is degraded relative to the consistent *tet*(G) measured in the background community, suggesting that the *E. coli* DNA degradation is not linked to the overall decline of biomass. In contrast, the MRSA DNA also shows some decline, but this trend is mirrored in the background *tet*(G) levels as well indicating that the trend is affecting the digester biomass as a whole, such as through slow attrition of the digester biomass under the imposed starvation conditions (batch reactors were not "fed" raw sludge during the experiment) or DNA extraction differences between samples (later time points (>30 days) were extracted in a different batch than earlier samples). Alternately, the difference between slow degradation of *E. coli* DNA and relative persistence of MRSA DNA may be linked to a potential residual strength of the Gram positive cell wall over a Gram negative cell wall in the degradation of dead cells or the differential survival of *E. coli* and MRSA in their respective VBNC states. It is interesting to compare the fate of MRSA DNA in sludge at 53 °C to 37 °C, specifically the time lag between the extinction of plate counts and the onset of DNA degradation. In 53 °C, MRSA DNA does not show significant degradation relative to background DNA, whereas at 37 °C, MRSA DNA degraded within 20 days. This also seems to support the formation of a VBNC state in MRSA, with a slower attrition rate (i.e., more recalcitrant VBNC state) than *E. coli*.

A comparison of abiotic conditions and a speculation on the indigenous biological activity in the two temperature regimes is warranted. The starting pH of all studies was in the range of 7.2 to 7.7; however, the pH was not monitored during the studies except for the extended thermophilic digestions of *E. coli* and MRSA, where pH 8.4 was measured on Day 21 (out of Day 60). Due to lack of data, it is difficult to conclude whether pH played a role in the different degradation rates of DNA, but DNA degradation under various sludge pH regimes warrants further study. Alternately, protozoa are known to prey upon bacteria in aqueous environments (Gurijala et al., 1990; Scheurman et al., 1988), but protozoa are also susceptible to thermal inactivation (Wichuk et al., 2007). However, it would be expected that simultaneous disappearance of plate counts and DNA would result from predation/engulfment, so in these experiments, predation does not appear to be a factor.

The relative survival of extracellular DNA in thermophilic digested sludge could also be explained by a less active biomass with respect to the degradation and uptake of lysed pathogen DNA. However, the pathogen gene concentrations in an aliquot of sample treated with DNase to

degrade extracellular DNA were not significantly lower than an untreated aliquot. A positive control (addition of extracellular DNA) was attempted to assess the extent of the DNase treatment, but the extracellular DNA was below the LOQ of QPCR in untreated (no DNase) samples, thus the effectiveness of the DNase treatment in degrading extracellular DNA could not be directly quantified or verified. Difficulties in recovering spiked extracellular DNA has been reported by others, with recovered amounts less than 6% of the added amounts (Pietramellara et al., 2009). Breazeal et al. (2013) reported that extracellular DNA bound to wastewater colloids was susceptible to DNase degradation. However, other studies have shown that extracellular DNA is protected from DNase degradation when bound by humic acids (Crecchio et al., 1998), organic matter (Nguyen et al., 2010), and river sediments, specifically clay (Mao et al., 2014). Nielsen et al. (1996) reported that the DNA concentrations found in extra polymeric substances (EPS) composing sludge flocs remained stable throughout 12 day storage of activated sludge under anaerobic conditions, while the concentrations of protein and carbohydrates were greatly reduced. Future work should consider methods to ascertain the proportions of intracellular and extracellular DNA (Mao et al., 2014). There would be a difference between the fate of extracellular DNA and DNA contained within dead, unviable cells. DNA contained within nonviable cells would be protected from horizontal gene transfer via conjugation (requires live cells) or transformation (uptake of free extracellular DNA). While it is expected that dead cells would eventually lyse and release DNA into the surrounding environment, the rate of lysis relative to the rate of cell death has not been investigated.

As such, the combination of results suggests that the recalcitrance of DNA in high temperature sludge treatments is caused by *E. coli* and MRSA entering a VBNC state, in which the culturability of the pathogen is impacted, but the DNA and cellular components remain intact and the cell is theoretically capable of resuscitation. In general, the Gram positive cell wall of MRSA may slow the lysis process and provide an additional barrier to subsequent DNA degradation relative to Gram negative *E. coli*.

In addition to the relative survival of pathogens in sludge treatment, the opportunities for horizontal gene transfer resulting in the persistence and release of ARGs to the environment is worth evaluating. In similar experiments with tetracycline-resistant microbes isolated from the indigenous digester population and reintroduced at high concentrations (Chapter 3), evidence for

the transfer of ARGs between the spiked microorganism and the digester microbial population was observed. However, in all experiments with *E. coli* SMS-3-5 and MRSA, the chromosomal pathogen gene (*nuc* and *gad*A/B) trends were similar in concentration and trend to the pathogen-associated ARGs contained on transferable mobile genetic elements. Likewise, pathogen ARG concentrations either disappeared following plate counts while indigenous ARG concentrations remained constant (20 °C and 37 °C) or were persistent with the conclusion that the host ARB was persistent to some extent (*E. coli*) or great extent (MRSA) in the VBNC state (53 °C, thermophilic digestion).

Conclusions

Improved understanding of pathogen inactivation kinetics and mechanisms is required to improve design and operation of sludge stabilization technologies, evaluate potential adverse health risks, increase public perception and confidence in their local wastewater utilities, and to abate the dispersion of ARGs and clinically relevant pathogens associated with the land application of biosolids. In this study, the differential survival of MRSA and *E. coli* in sludge treatments as monitored with plate counts (culturing), QPCR, and live/dead assay highlights the variability among inactivation and DNA persistence among a wide range of pathogens based on thermotolerance, cell wall composition, and matrix conditions.

This study investigated the inactivation kinetics and mechanisms of MRSA and *E. coli* SMS-3-5 in conditions simulating land application, mesophilic digestion, thermophilic digestion, and pasteurization. *E. coli* exhibited an elevated persistence relative to MRSA as measured by plate counts and DNA in sludge experiments representing land application and mesophilic digestion, suggesting that *E. coli* is more tolerant of abiotic and biotic conditions of digested sludge. DNA persistence despite loss of culturability under thermophilic digester conditions suggest that both *E. coli* and MRSA enter a viable but nonculturable state. Extended experiment times as well as additional analytical methods geared toward differentiating the relative contributions of intracellular DNA, extracellular DNA, and DNA housed within viable, VBNC, or dead but unlysed cells is required to further assess inactivation mechanisms in thermophilic digestion and pasteurization.

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Figures

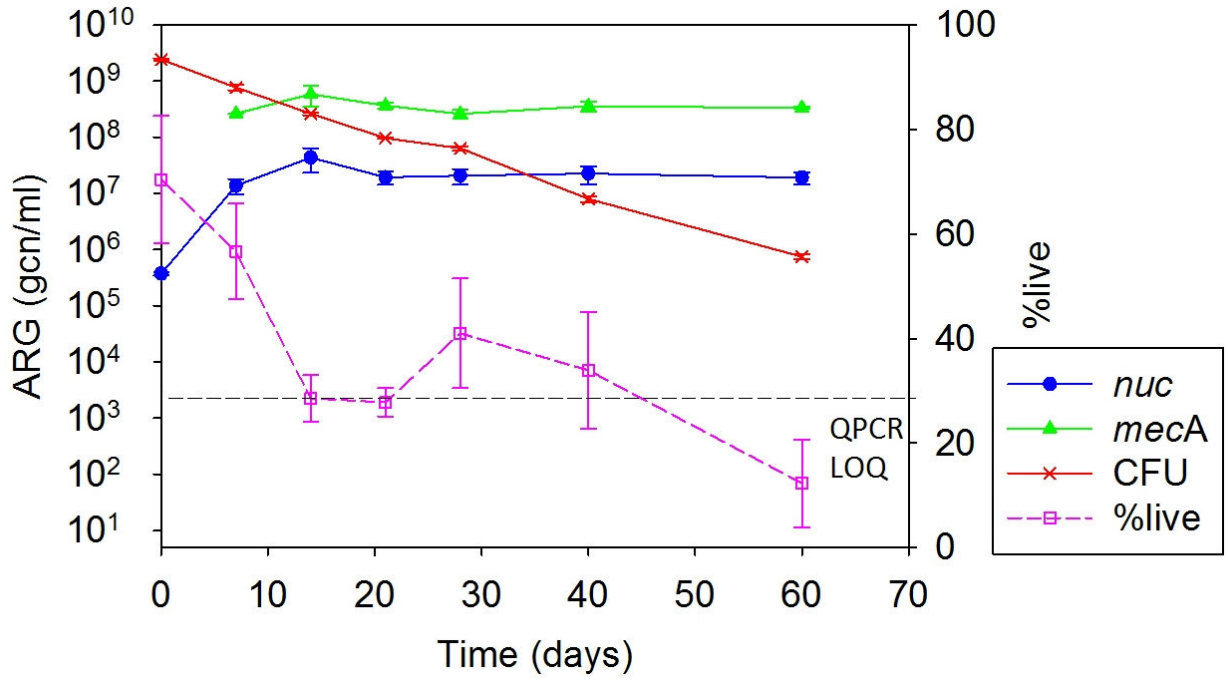

Figure 2-1. Plate counts (CFU/ml PBS), ARG (*mec*A) and MRSA-specific gene (*nuc*) (gene copy numbers (gcn/ml PBS) for MRSA at 20 °C in PBS.

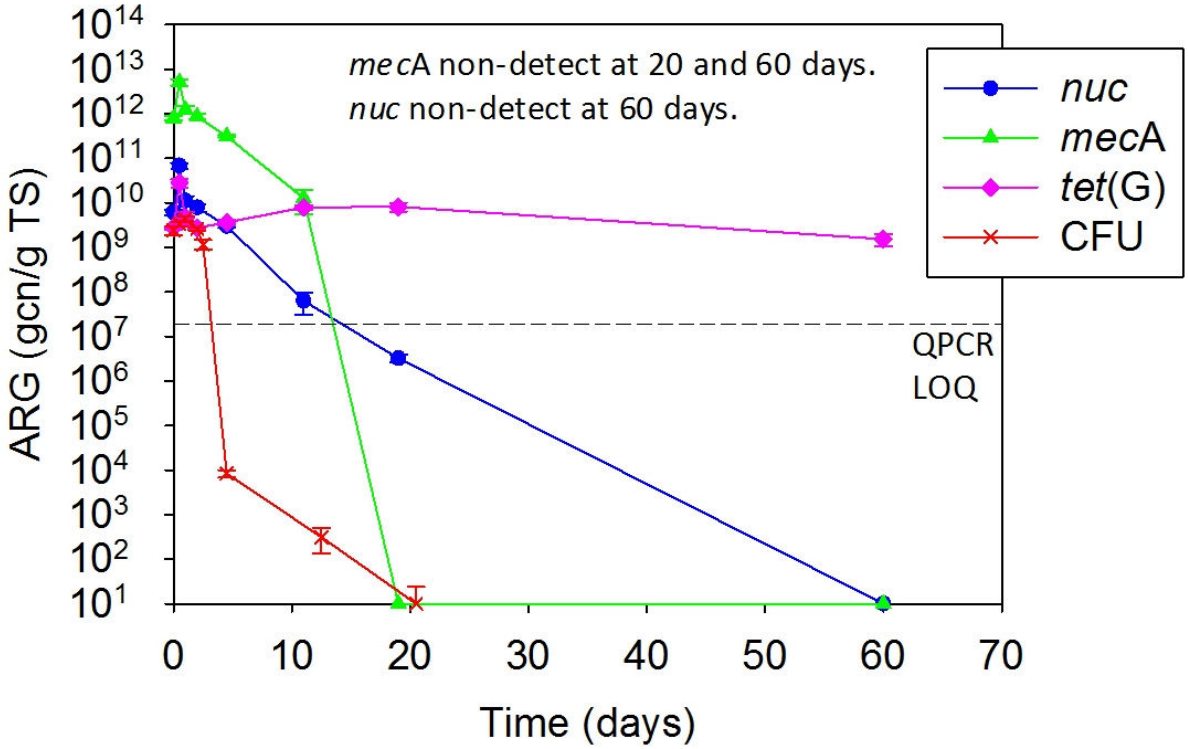

Figure 2-2. Plate counts (CFU/g TS), ARGs (*mec*A, *tet*(G)) and MRSA-specific gene (*nuc*) (gene copy numbers (gcn/g TS) for MRSA at 20 °C in digested sludge.

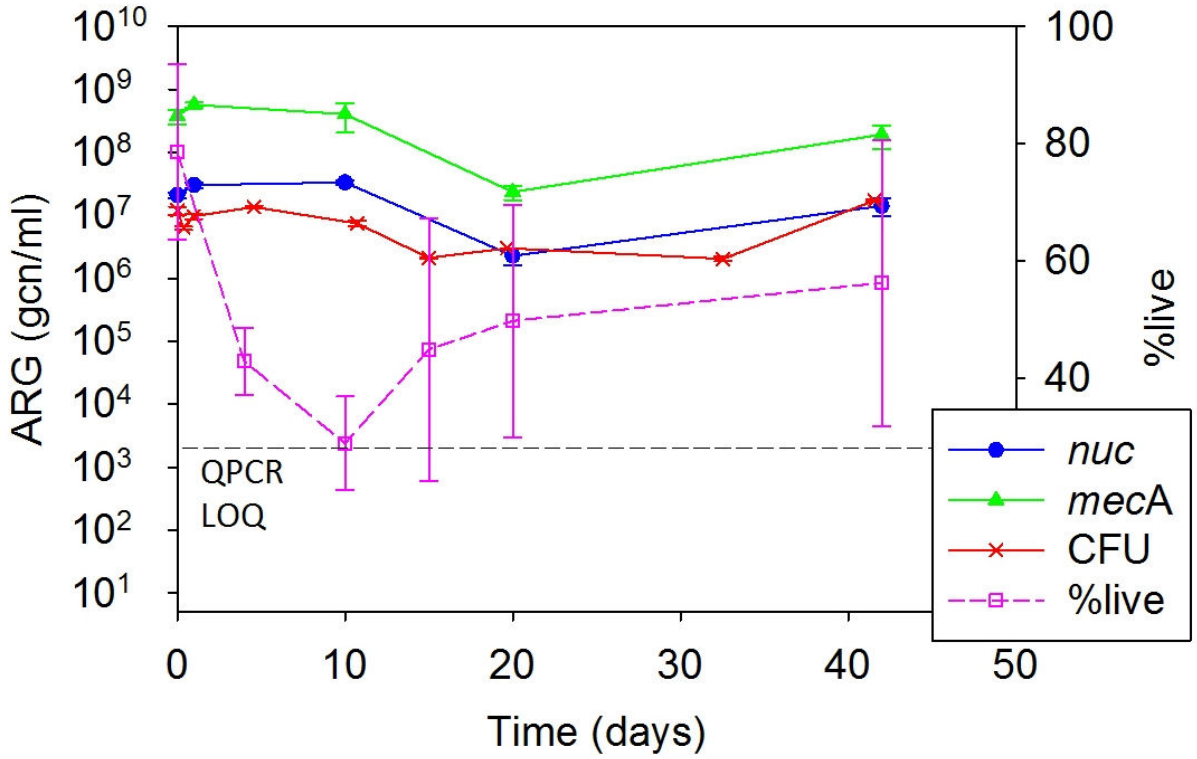

Figure 2-3. Plate counts (CFU/ml PBS), ARG (*mec*A) and MRSA-specific gene (*nuc*) (gene copy numbers (gcn/ml PBS) for MRSA at 37 °C in PBS.

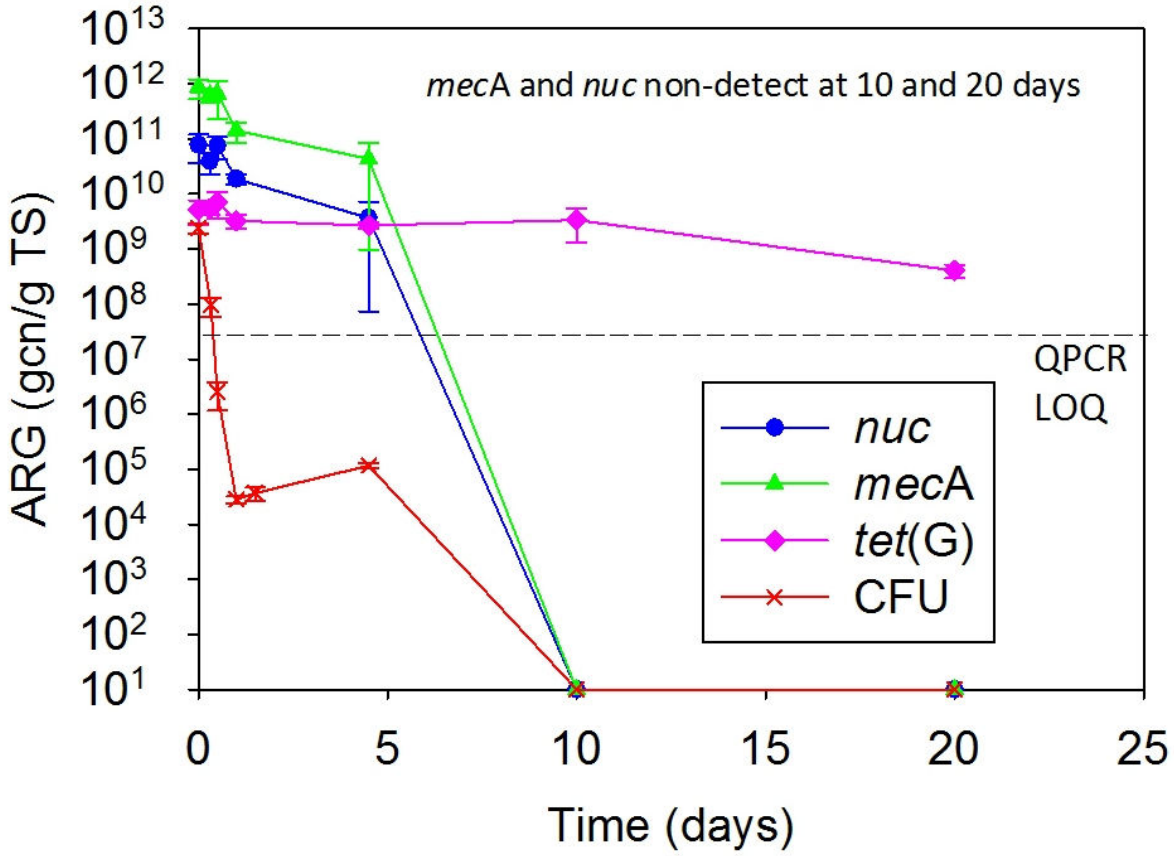

Figure 2-4. Plate counts (CFU/g TS), ARGs (*mec*A, *tet*(G)) and MRSA-specific gene (*nuc*) (gene copy numbers (gcn/g TS) for MRSA at 37 °C in digested sludge.

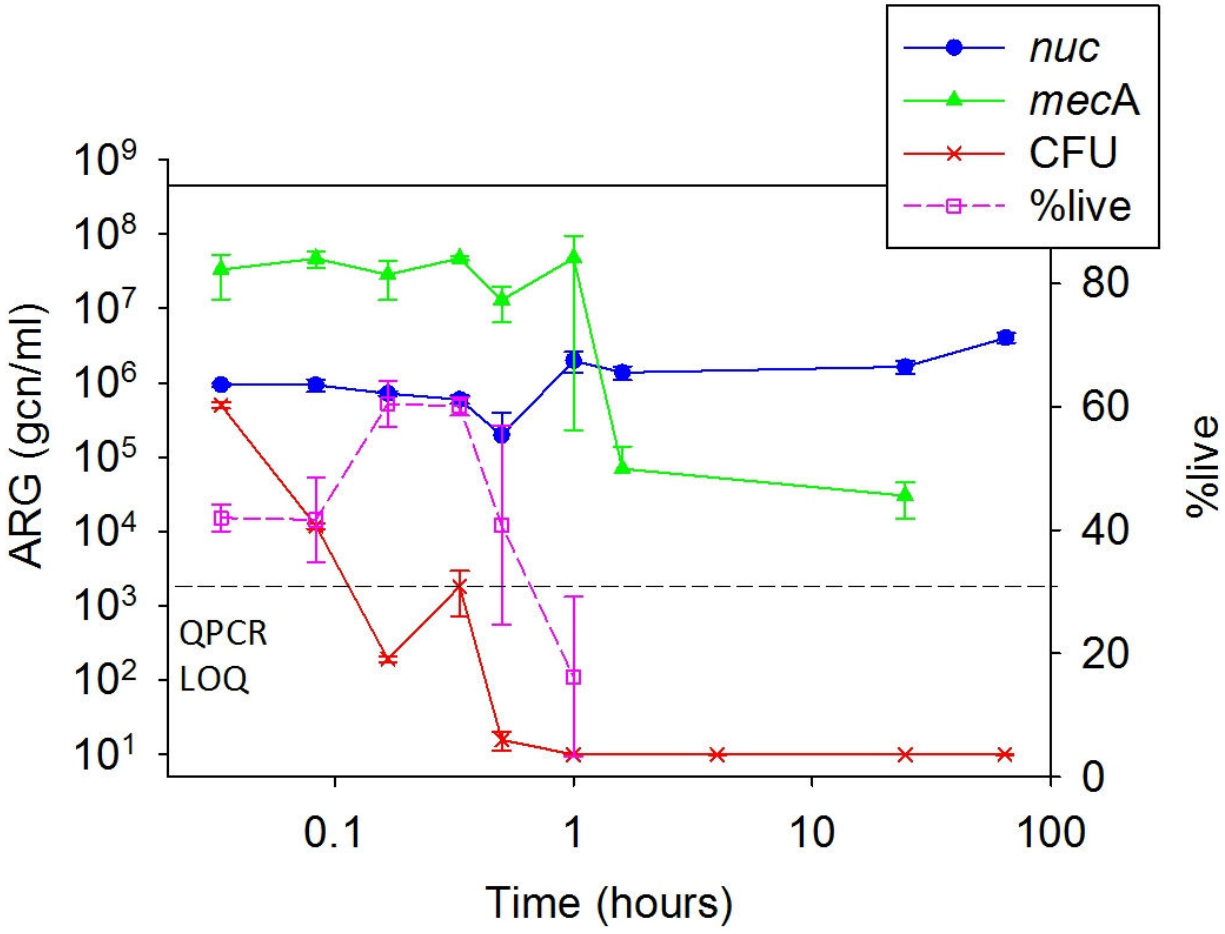

Figure 2-5. Plate counts (CFU/ml PBS), ARG (*mec*A) and MRSA-specific gene (*nuc*) (gene copy numbers (gcn/ml PBS) for MRSA at 53 °C in PBS.

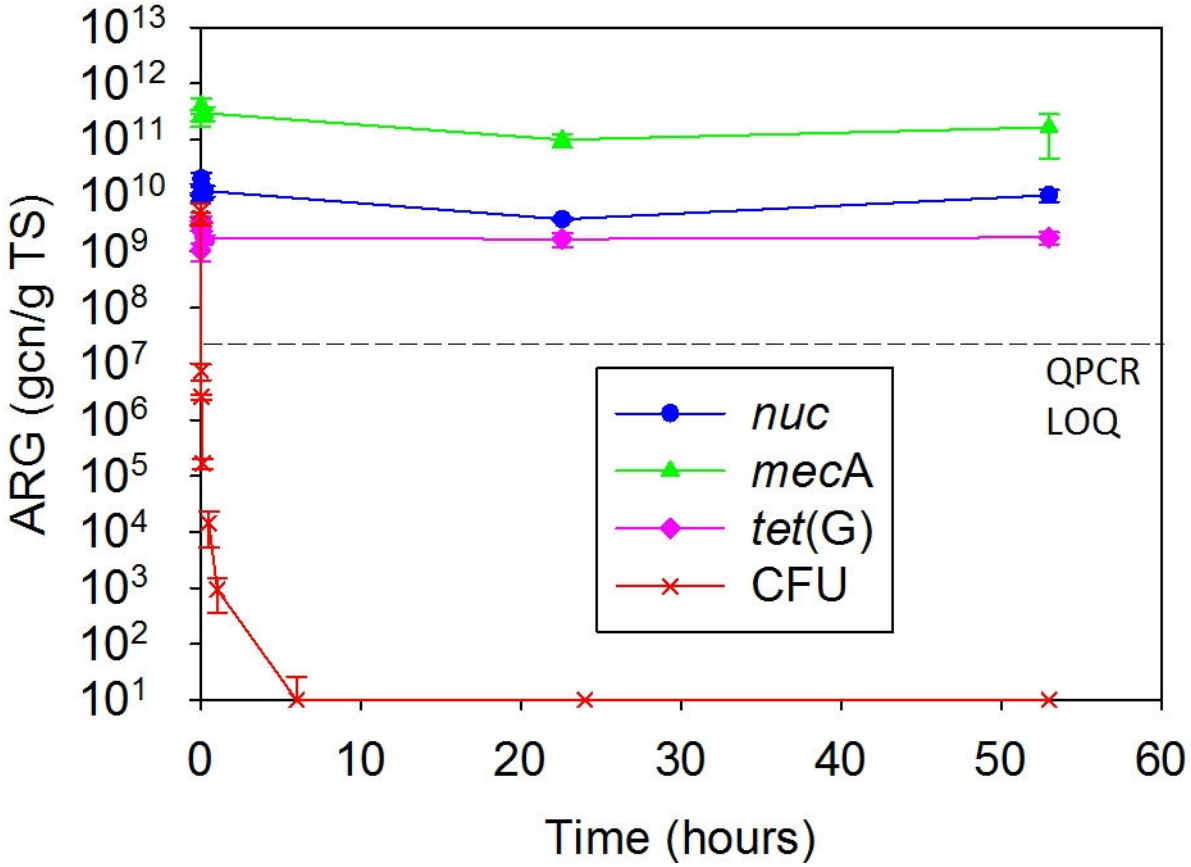

Figure 2-6. Plate counts (CFU/g TS), ARGs (*mec*A, *tet*(G)) and MRSA-specific gene (*nuc*) (gene copy numbers (gcn/g TS) for MRSA at 53 °C in digested sludge.

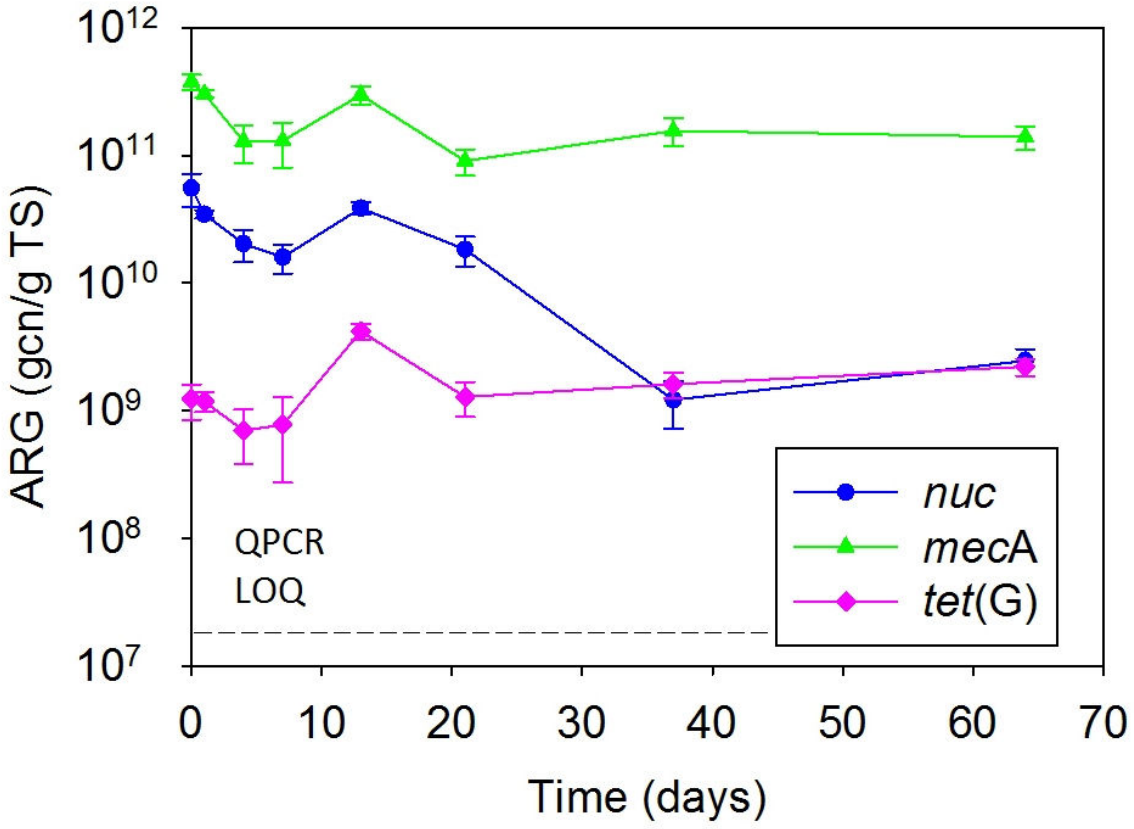

Figure 2-7. ARGs (*mec*A, *tet*(G)) and MRSA-specific gene (*nuc*) (gene copy numbers (gcn/g TS) for MRSA at 53 °C in digested sludge for an extended experiment length of 60 days.

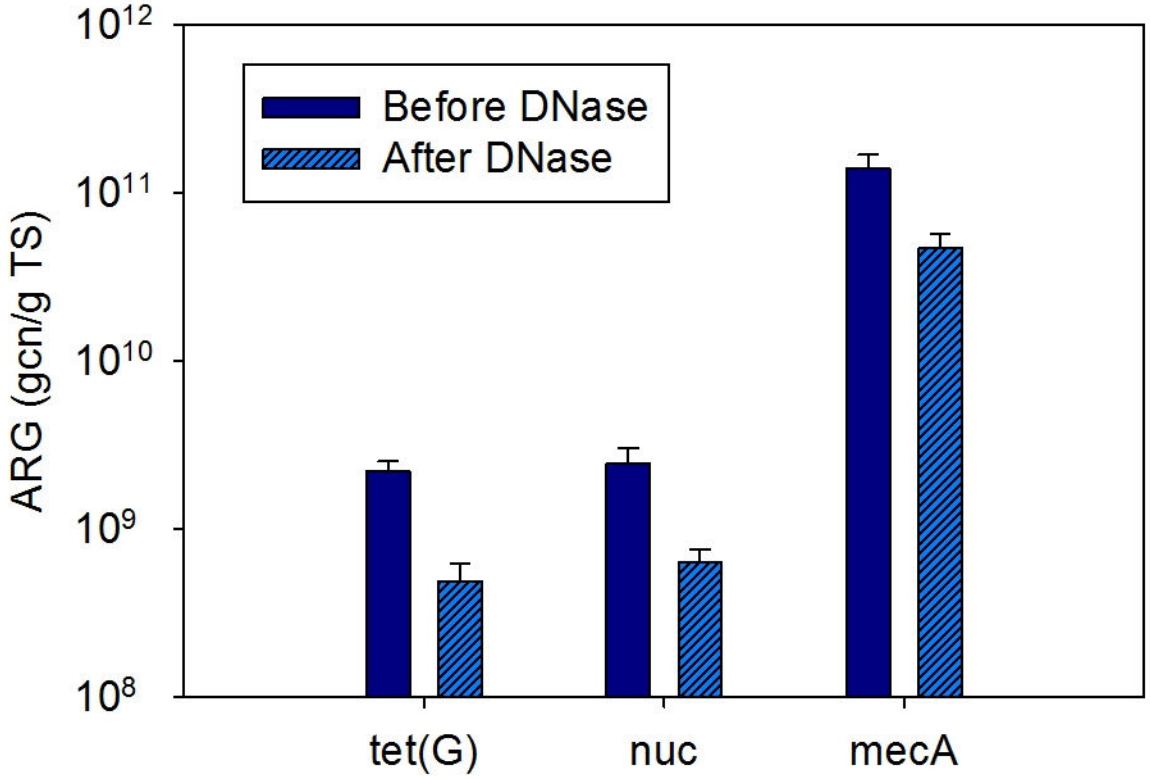

Figure 2-8. Comparison of ARGs (*mec*A, *tet*(G)) and MRSA-specific gene (*nuc*) (gene copy numbers (gcn/g TS) in Day 60 samples for MRSA at 53 °C in digested sludge for an extended experiment length of 60 days.

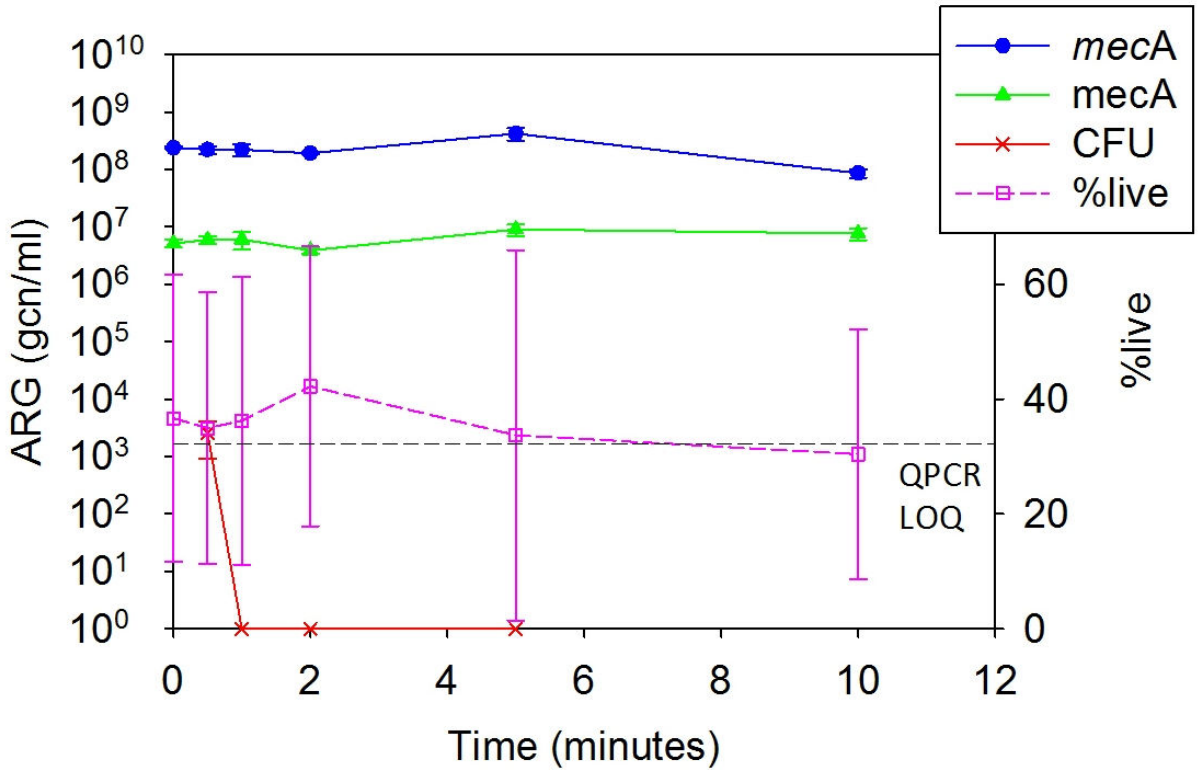

Figure 2-9. Plate counts (CFU/ml PBS), ARG (*mec*A) and MRSA-specific gene (*nuc*) (gene copy numbers (gcn/ml PBS) for MRSA at 70 °C in PBS.

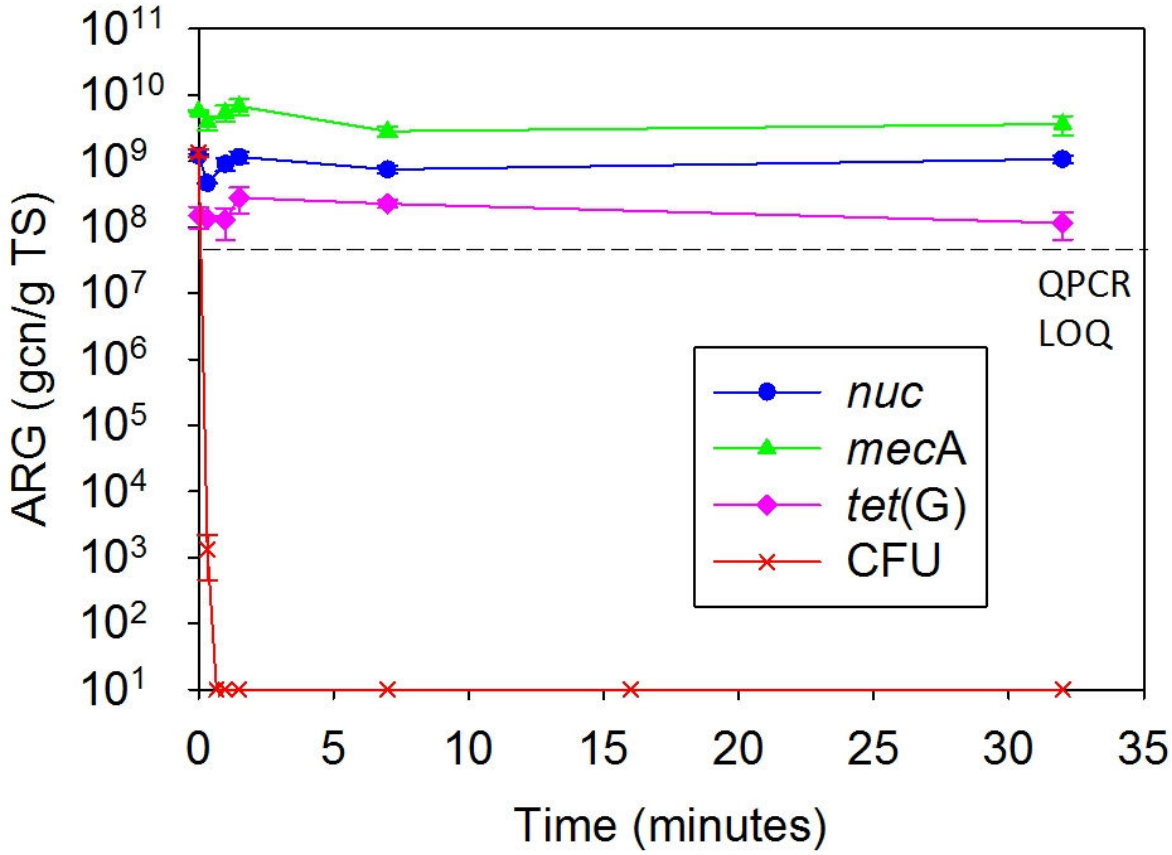

Figure 2-10. Plate counts (CFU/g TS), ARGs (*mec*A, *tet*(G)) and MRSA-specific gene (*nuc*) (gene copy numbers (gcn/g TS) for MRSA at 70 °C in digested sludge.

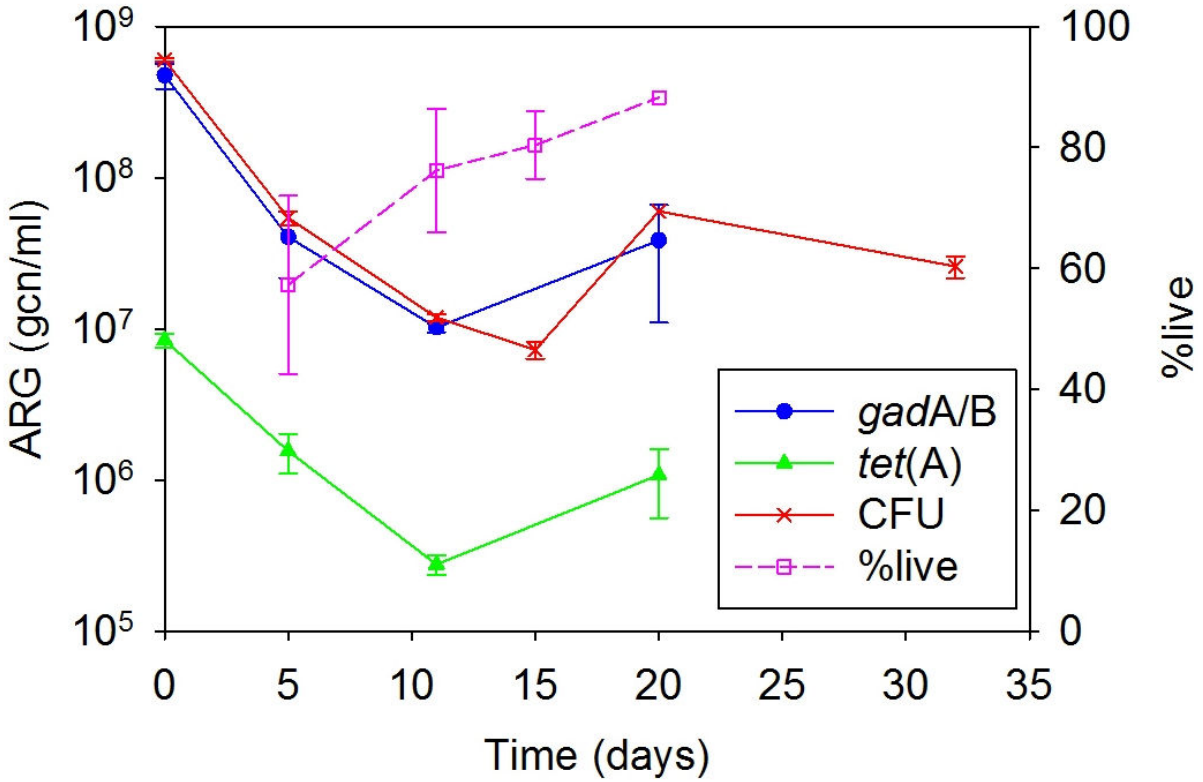

Figure 2-11. Plate counts (CFU/ml PBS), ARGs (*tet*(A), *sul*2, *tet*(G)) and *E. coli*-specific gene (*gad*A/B) (gene copy numbers (gcn/ml PBS) for *E. coli* at 37 °C in PBS.

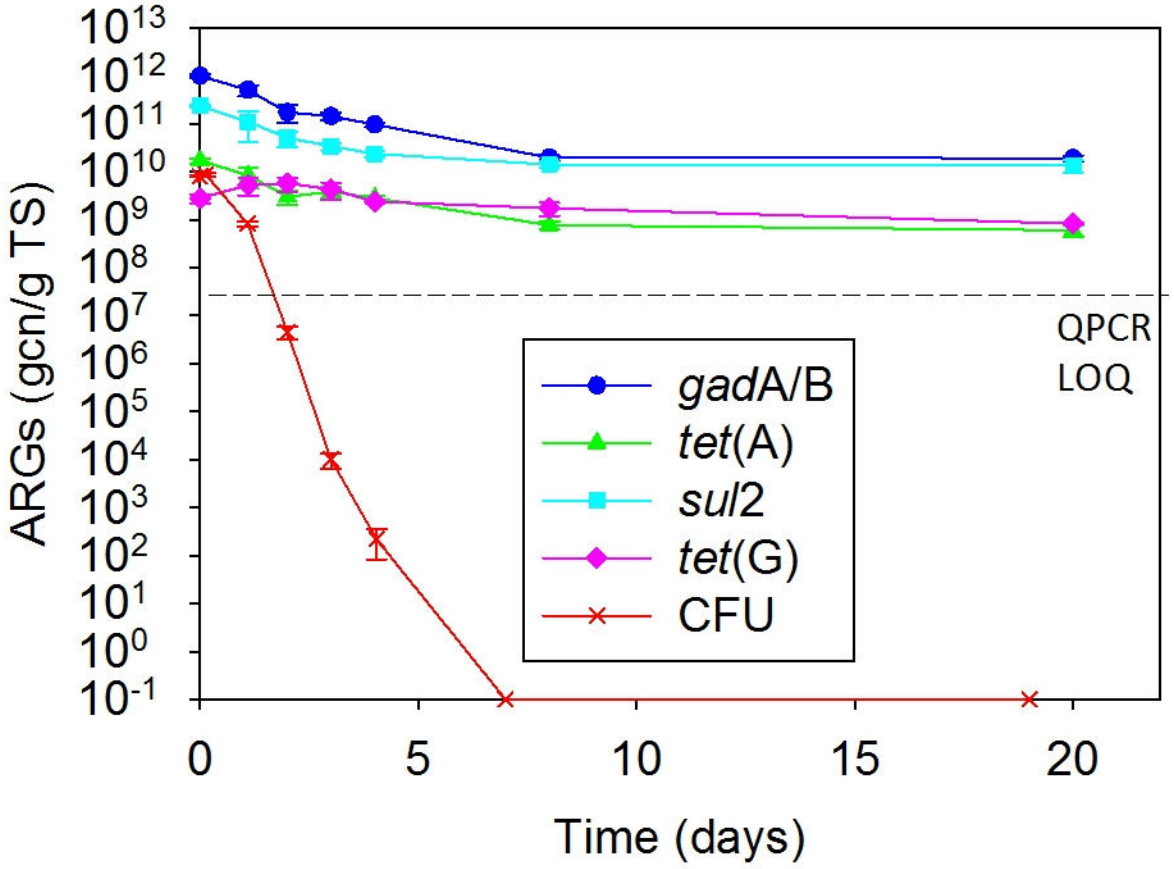

Figure 2-12. Plate counts (CFU/g TS), ARGs (*tet*(A), *sul*2, *tet*(G)) and *E. coli*-specific gene (*gad*A/B) (gene copy numbers (gcn/g TS) for *E. coli* at 37 °C in digested sludge.

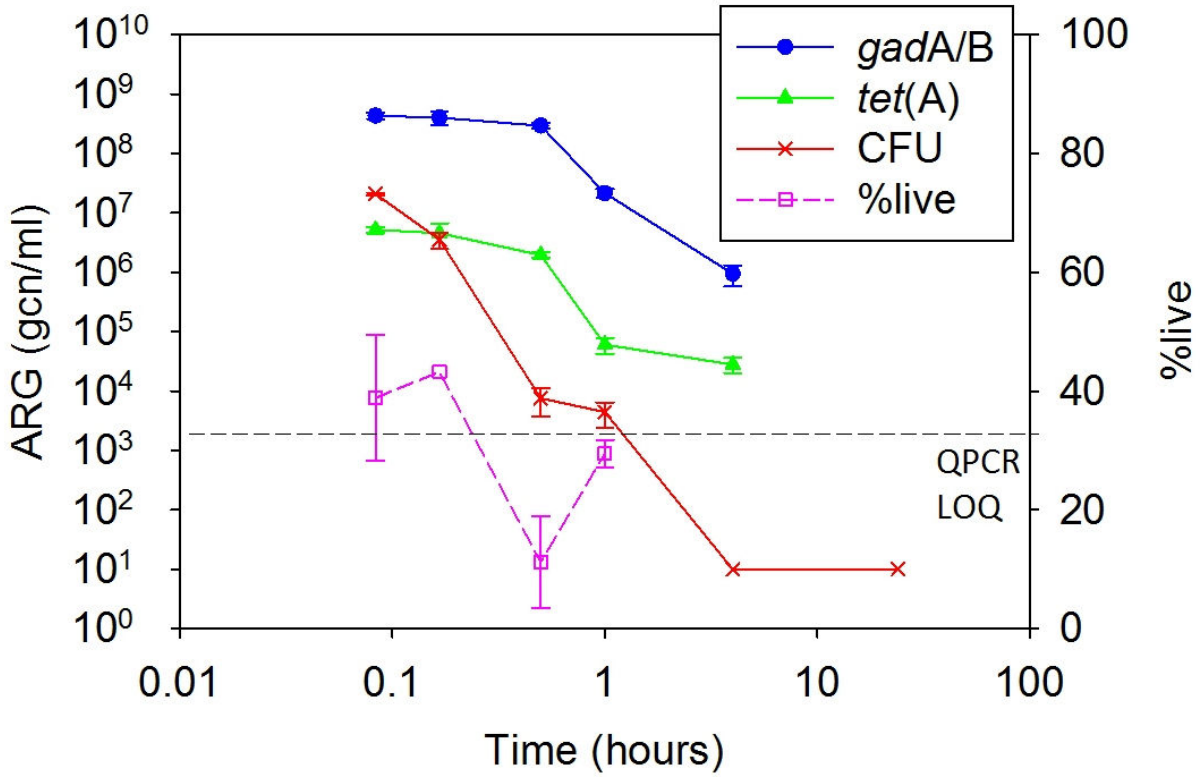

Figure 2-13. Plate counts (CFU/ml PBS), ARGs (*tet*(A)*, sul*2, *tet*(G)) and *E. coli*-specific gene (*gad*A/B) (gcn/ml PBS) for *E. coli* at 53 °C in PBS.

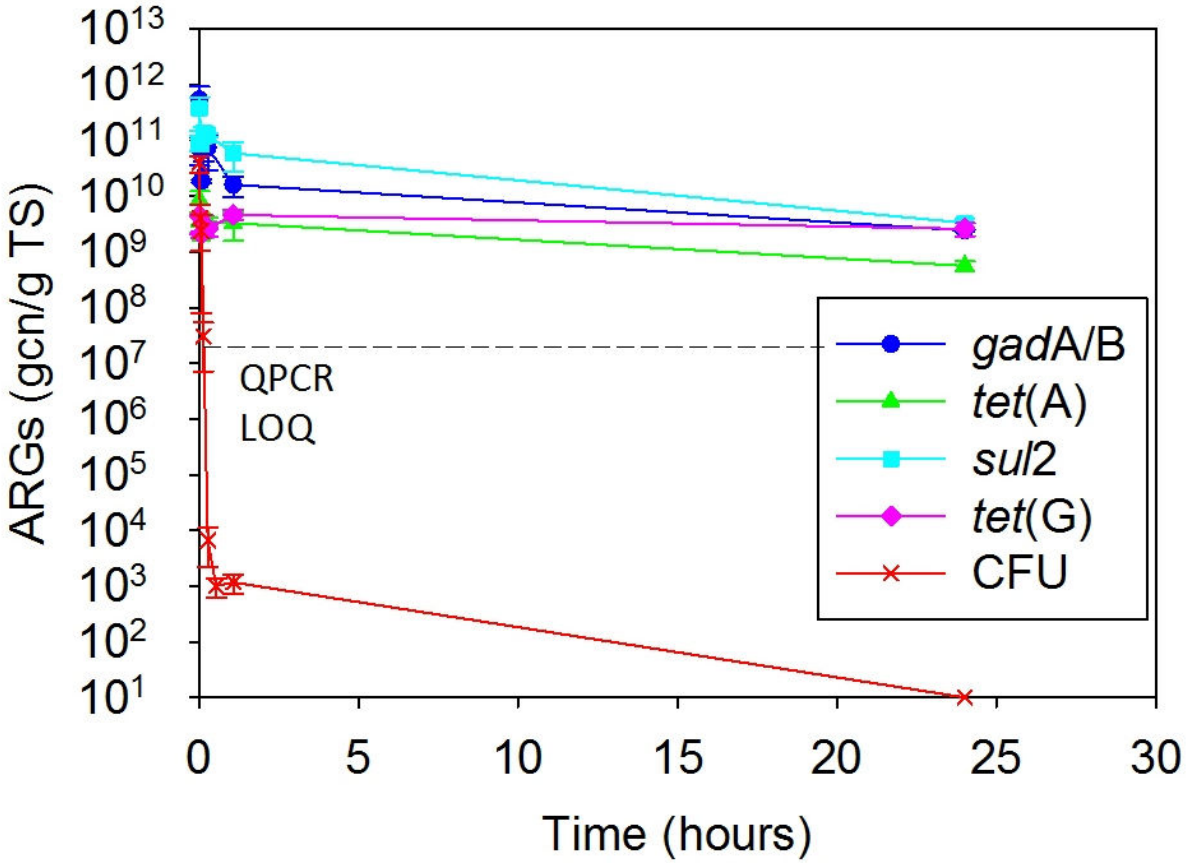

Figure 2-14. Plate counts (CFU/g TS), ARGs (*tet*(A), *sul*2, *tet*(G)) and *E. coli*-specific gene (*gad*A/B) (gene copy numbers (gcn/g TS) for *E. coli* at 53 °C in digested sludge.

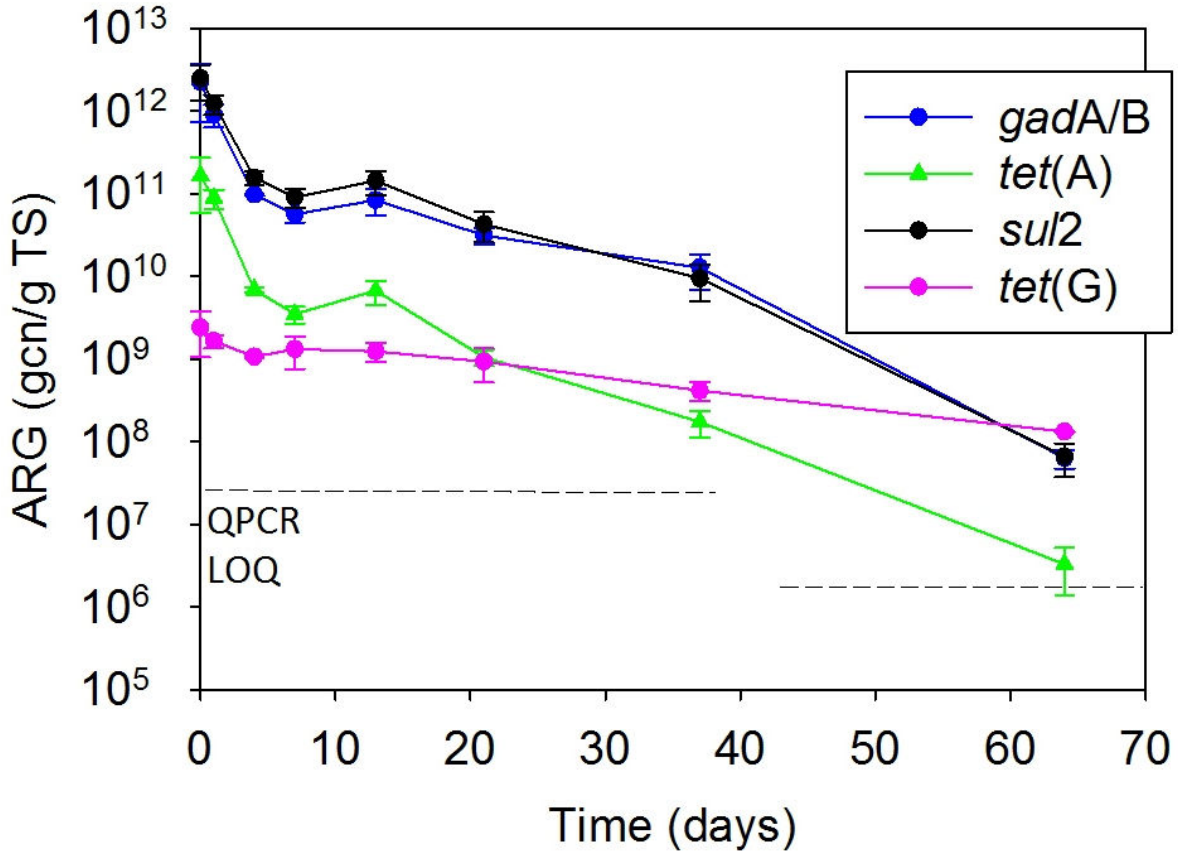

Figure 2-15. ARGs (*tet*(A), *sul*2, *tet*(G)) and *E. coli*-specific gene (*gad*A/B) (gene copy numbers (gcn/g TS) for *E. coli* at 53 °C in digested sludge for an extended experiment length of 60 days. The 60-day LOQ changed from previous time points because DNA was extracted from a smaller volume of sample.

CHAPTER 3

Mechanisms of ARG Persistence and Attenuation During Anaerobic Digestion

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Keywords: Mesophilic anaerobic digestion, thermophilic anaerobic digestion, antibiotic resistance gene, horizontal gene transfer

Introduction

Bacterial resistance to antibiotics is a worldwide problem resulting in untreatable infections, death, and escalating healthcare costs. The connection between antibiotic abuse in human medicine and agricultural settings with antibiotic resistance is well established, although prevention of the continued spread of resistance is less clear. The landscape of the war against antibiotic resistance is expanding from hospitals and clinical settings to environmental reservoirs, particularly those reservoirs that serve as conduits from anthropogenic or agricultural sources to the environment, such as wastewater treatment plants. Numerous studies have presented findings showing gene transfer between commensal (environmental) bacteria and pathogen bacteria of clinical significance (Poirel et al., 2002; Rossolini et al., 2008; Szczepanowski et al., 2009).

Bacterial resistance to antibiotics arose in nature and it seems possible that some mitigation efforts may also be rooted in nature. Addressing the environmental reservoirs of antibiotic resistance may be part of the solution to abate or attenuate the spread of antibiotic resistance between clinically-relevant microorganisms and environmental microorganisms (Davison, 1999; Wellington, 2013). Wastewater treatment plants have been identified as hotspots of propagation and transfer of ARGs between bacteria as well as dissemination points of ARGs to the environment (Droge et al. 2000; Schluter et al. 2007). More pressing, Szczepanowski et al. (2009) has linked the occurrence of new ARGs in clinical bacteria to wastewater origin. The nutrient-rich, microbial-dense processes at a WWTP, in concert with the presence of antibiotic or other selection factors, has been shown to result in the persistence or enhancement of ARG concentrations in activated sludge (Stalder et al. (2013), wastewater effluent (Zhang, 2009); Ferreira da Silva (2006); Luczkiewicz, et al. (2010), and land-applied biosolids (Munir et al. (2011a), Munir et al. (2011b)). Early studies focused on the presence and quantification of

specific ARBs or ARGs in wastewater matrices while more recent studies have targeted the use of metagenomics to characterize and quantify the dissemination vehicles of ARGs, i.e., mobile genetic elements such as transposons, integrons, plasmids, and bacteriophages (Muniesa et al., 2011; Moura et al. 2012). These mobile genetic elements represent the agents of genetic transfer and ARG dissemination between bacteria. It has been argued that monitoring of mobile genetic elements rather than specific ARGs or ARBs is important toward quantifying and understanding the overall occurrence and spread of resistance within WWTPs (Lupo et al. 2012, Stalder, et al. 2012, Moura et al. 2012; Rizzo et al. 2013) and the environment (Ashbolt et al., 2013; Heuer and Smalla, 2012; Smalla and Sobecky, 2002).

While these studies have shown the diverse presence of mobile genetic elements and ARGs within wastewater matrices, deciphering the mechanisms of ARG persistence or attenuation in wastewater treatment processes and the environment as a whole remain unresolved. Furthermore, mechanistic studies targeting the meaningful reduction of genetic transfer processes, ARBs, or ARGs that may lead to resistance abatement is still lacking, particularly in sludge treatment.

To date, four published studies have investigated the effects of anaerobic digestion on ARGs, Ghosh et al. (2009), Diehl and LaPara (2010), Ma et al. (2011), and Miller et al. (2013). Collectively, these studies measured tetracycline, sulfonamide, erythromycin, and Class 1 integrase genes in the thermophilic and mesophilic digestion. Thermophilic digestion reduced all genes to some extent, whereas mesophilic digestion had variable effects on gene concentrations. Ma et al. (2011) also reported an enhanced removal of ARGs during thermal hydrolysis pretreatment. However, the concentration of ARGs increased, with the exception of *sul*1 and *tet*(G), in the subsequent mesophilic stage. These results suggest that the digester microbial community and the operating conditions that influence the development and maintenance of that community play an important role in determining ARG fate. Aside from temperature, digester operating conditions, digester ARG composition, and raw sludge ARG composition that might affect effluent (biosolid) ARG composition have not been investigated.

It seems appropriate to approach mechanistic studies of ARG fate by first considering the different factors that may influence ARG attenuation or persistence. ARG survival can be fixed

to the fate of the ARB housing the gene through replacement growth, selective enrichment, or death. Conjugation also plays a role through the association of ARGs with mobile genetic elements. Extracellular ARGs from lysed, dead ARBs or DNA expulsion and plasmid ejection may persist in the environment until degraded or until taken up by other bacteria via transformation. Mao et al. (2014) reports on the large reservoir of extracellular ARGs compared to intracellular ARGs in river sediments, reflecting the importance of DNA persistence outside of a host cell in the environment. Environmental conditions (pH, temperature, aerobic/anaerobic, nutrient status), stress, toxicity, predation, or selection pressures may also influence ARB fate and frequency of horizontal gene transfer events and extracellular DNA persistence.

It is difficult to measure horizontal gene transfer events in complex environmental matrices. Rizzo et al. (2013) summarized that there are three types of retrospective evidence of horizontal gene transfer, including association of the ARG with a mobile genetic element, loss of co-location of the insertion site in the host and the acquired ARG, or the lack of similarity between the ARG phylogeny and the ARB phylogeny. Microcosm studies in sediment (Bonot and Merlin, 2010), activated sludge, biofilm and anaerobic digestion (Merlin et al., 2011) used an increasing ratio of introduced plasmid DNA normalized to ARB chromosomal DNA over time to signify horizontal gene transfer between the introduced ARB and indigenous bacteria. Here, quantification of horizontal gene transfer in anaerobic digestion is approached by tracking the fate of the ARG via quantitative polymerase chain reaction (QPCR) and the fate of the donor ARB by plate culturing in batch digestion experiments.

Specifically, in this study, two tetracycline-resistant microorganisms were isolated from mesophilically-digested sludge (Iso M1-1) and thermophilically-digested sludge (Iso T10). [Note, isolate names are based on an arbitrary numbering system used in the isolation process. Isolate names do not correlate with any known strain or plasmid identification.] The isolates were grown in pure culture to density, concentrated in PBS, and spiked into batch anaerobic digesters to monitor the fate of the isolate (ARB) and tetracycline ARG with time using QPCR and plate culturing. In a separate experiment with semi-continuously fed digesters, the ARG concentrations of influent raw sludge and digested sludge were monitored in lab-scale mesophilic and thermophilic digesters over a 9-month period to evaluate the impact of influent raw sludge ARG composition on digester effluent ARG composition.

Materials and Methods

Culture Plates. Tryptic soy agar (TSA, 40 g/L) was added to reverse osmosis water, dissolved by heating, and autoclaved at 121 ºC for 20 minutes. The agar was cooled to 55 ºC in a 55 ºC water bath. Filter-sterilized solutions of tetracycline hydrochloride (Sigma Aldrich) and cycloheximide (Sigma Aldrich) were added to the agar at final concentrations of 16 mg/L and 200 mg/L, respectively. Agar was mixed at low speed on a stir plate prior to pouring petri dishes (100mmx15mm). These plates were used in the isolation of tetracycline-resistant microorganisms as well as batch digestion experiments.

Culture Tube Experiments, Isolation of Tetracycline-resistant Microorganisms. Tetracycline-amended (16 µg/ml) TSA plates were inoculated with 100 ul of 1x, 10x, and 100x diluted mesophilic or thermophilic digested sludge and incubated at 37 ◦C for 24 hours. After 24 hours, an individual colony was aseptically re-streaked on a clean tetracycline-amended TSA plate and incubated again for 24 hours. This process was repeated for at least 3 times for 5 colonies (mesophilic) and 13 colonies (thermophilic). After this initial isolation step, colonies had the same morphological appearance on agar plates and growth curves in tryptic soy broth. Growth curves of the isolates in tryptic soy broth yielded a 6 hour growth time (to stationary phase) for the mesophilic isolate (Iso M1-1) and a 10 hour growth time (to stationary phase) for the thermophilic isolate (Iso T10). QPCR analysis *tet*(C), *tet*(G), *tet*(O), and *tet*(W) on DNA extracts of dense pure culture broth samples of all isolates revealed that Iso M1-1 was positive only for *tet*(G) and Iso T10 was positive only for *tet*(W). None of the other tested isolates were positive for *tet*(G), *tet*(W), *tet*(C), or *tet*(O). Freezer stock of the isolates was made with cultures grown to density, concentrated by centrifugation, rinsed with phosphate buffered saline, re-concentrated, resuspended in tryptic soy broth with 20% glycerol, and frozen at -80 ºC until use.

For experiments, six 100 ml flasks of tryptic soy broth were spiked with 1ml of freezer stock each and grown to late log phase. Cells were harvested by centrifugation at 10,000 rpm for 10 minutes and re-suspended and rinsed in phosphate buffered saline. The rinse step was repeated three times prior to final resuspension in PBS (~30 ml total). The absorbance at 600nm was measured for a 10x dilution of the concentrated cell suspension. An absorbance of 1.2 approximated a cell density of 10^8 cells per ml (10^9 cells per ml in the undiluted suspension).

Experimental Setup. Digested sludge (4.5ml) was placed in plastic disposable culture tubes (100x17mm, Fisher Scientific) and placed in a water bath at the test temperature (i.e., 37 °C or 53 °C). Three replicates were unamended and served as a control blank. Other replicates received a spike of the concentrated pure culture of tetracycline-resistant isolate. Over a range of time points up to 40 days suitable for capturing the death/decay curve, triplicate sample tubes were removed and immersed in a water/ice bath to quench the temperature reaction. After quenching, plating was done immediately and an aliquot of the sample was stored at -20 ◦C until DNA extraction.

Cell Culturing/Plating. Plating of cells was done immediately by serial 10x dilutions of the original sample and inoculating two tetracycline-amended TSA plates with 100 ul of diluted sample. Prior to each dilution and plating, culture tubes were vortexed a moderately high speed for 30 seconds to disengage bacteria from sludge particles. Inadequate vortexing resulted in clustered colonies. Inoculated plates were incubated at 37 °C for 24 hours prior to cell count. Background cells counts were on the order of $1.3x10^3$ cells per ml for the mesophilic study and $7.3x10^2$ cells per ml for the thermophilic study. At these dilution levels, a few different cell morphologies were evident, but it was not possible to distinguish the Iso M1-1 and Iso T10 colonies from other background tetracycline-resistant bacteria.

Quantification of ARGs. DNA was extracted from 175 µL of digested sludge from the culture tube samples using the MagMax Total Nucleic Acid Extraction Kit (Ambion, Life Technologies) according to manufacturer's protocol. Extracted DNA was diluted 50× to minimize inhibitory effects as determined by analyzing various dilutions of DNA extract. Diluted DNA extracts were stored at -20°C until analysis by QPCR for bacterial 16S rRNA genes, *tet*(G), and *tet*(W). A 10 µL reaction mixture was comprised of 5.0 µL SsoFast Evagreen Supermix (Bio-Rad, Hercules, California), 0.8 µL of each 5 µM primer (Ma et al., 2011), 2.4 µl molecular biology grade water, and 1 µL of DNA template. All samples were quantified in triplicate. Standards prepared from serial dilutions of cloned genes ranged from 10^1 to 10^7 gene copies per µL. Standards and a reagent blank were included with each QPCR well plate. It is important to note that ARG gene copy numbers as plotted are a result of adjustment by three multiplication factors, including factors related to extraction of DNA from the sludge sample (0.3x), dilution of the extract to reduce QPCR inhibitor concentrations (50x), and unit conversion from ul to ml (1000x). Based

on this, the lowest standard on the QPCR curve (10 gcn per ul) would translate to $1.5x10^5$ gcn per ml digested sludge.

pH and TS/VS. Total and volatile solids (Method 2540-G) and pH (Method 4500) were analyzed at the start and end of the studies as specified in Standard Methods for the Examination of Water and Wastewater (APHA, 1995).

Continuous Feed Digester Operations/Experiments. One 15 L, 12-day solids retention time (SRT) thermophilic (53 °C) digester and one 10 L, 20-day SRT mesophilic (37 °C) digester were fed raw sludge daily. These digesters served as control digesters for other work and details regarding digester operation can be found elsewhere (Miller et al., 2013). In brief, high density polyethylene cone fermenters (Hobby Beverage Equipment Company, Temecula, California) with nominal volumes of 6.5 gallons (24.6 liters) were fed daily with a 70% primary sludge and 30% thickened waste activated sludge mixture from the Christiansburg, Virginia WWTP, which predominantly receives residential wastewater with minimal industrial contribution and no hospital contributions. This mixture was diluted with tap water to 2.5% total solids (TS) to maintain a consistent feed to the digesters to reduce variations in operating parameters (e.g., pH, alkalinity, VFAs, volatile solids reduction). During this study, raw sludge was collected from the plant every four to six weeks and stored at 4°C until fed into the digesters. Total solids (TS), volatile solids (VS), and pH of the raw sludge were routinely measured to ensure consistency of the feed sludge over the study period. A peristaltic pump was used to mix digester contents by recycling headspace gas to the bottom of the cone digester. Evolved gases were collected in 25-liter Tedlar bags (SKC, Inc., Eighty Four, Pennsylvania). Both digesters were maintained in a 37 ºC constant temperature room.

The mesophilic anaerobic digester was operated at an active volume of 10 L (2.6 gallons), 20-day SRT, with heating to 37 °C achieved from ambient conditions in a constant temperature room. The digester was initiated using 5 L of digested sludge seed from the Christiansburg WWTP. One-half L of feed sludge mixture was added per day until the 10L operating volume was reached. The thermophilic anaerobic digester was operated at an active volume of 15 L, 12-day SRT, and heated to a target temperature of 53 °C using electric heating tape with a temperature controller (Model No. BSAT 101-100, Thermolyne, Dubuque, Iowa). The

thermophilic digester was seeded with digested sludge from laboratory thermophilic anaerobic digesters (temperatures ranging from 48 °C to 57 °C) fed with primary and secondary solids from DC Water Blue Plains Advanced Wastewater Treatment Plant. The start-up of mesophilic and thermophilic digesters took place over a three-month period prior to the data collection presented herein and data collection spanned another nine months. Miller et al. (2013) reports data associated with the digester performance monitoring during the period of this study, including total and volatile solids, total alkalinity, pH, gas volume, headspace methane and carbon dioxide, and volatile fatty acids.

Results

Fate of Tetracycline Resistant Isolate (Iso M1-1) during Mesophilic Digestion. Iso M1-1 is a tetracycline-resistant microorganism containing *tet*(G) that was originally isolated from mesophilic (37 °C) digested sludge. As described in the experimental design, a concentrated culture of Iso M1-1 was spiked into culture tubes containing mesophilic digested sludge and incubated at 37 °C for 40 days. Cell counts, *tet*(G) (associated with Iso M1-1), and *tet*(W) (present in the background digester community) were monitored over the course of the experiment (Figure 1). The results of QPCR analysis are also provided as ARG copy numbers normalized to 16S bacterial rRNA (ARG ratio) (Figure 2). Normalization accounts for variability in DNA extraction efficiencies and biomass between samples. ARG ratios can be thought of as roughly representing an average gene count per bacteria cell and as such, can mask increases or decreases in un-normalized ARG copy numbers as a result of biomass growth or death. In addition, *tet(*G) was normalized to Iso M1-1 CFU plate counts to track the ratio of ARG to the original host ARB (Figure 3).

The background plate count of mesophilic sludge on tetracycline-amended tryptic soy agar plates was 1.3×10^3 CFU/ml (Figure 1). The plate count in initial spiked samples was 9.7×10^8 CFU/ml. The plate counts decreased over a period of 5 days and then remained at approximately 10^4 CFU/ml, or one-log above background, through the rest of the experiment.

The *tet*(G) content of the background digester community was 9.1×10^5 gcn/ml (shown as "*tet*(G) background" in Figure 1). Digester *tet*(G) content was raised to 2.9×10^7 gcn/ml after addition of concentrated Iso M1-1 (3.4×10^7 gcn/ml PBS). *tet*(G) normalized to 16S rRNA ratio is

0.28 in the pure culture PBS sample, suggesting that at least 28% of the population contains the *tet*(G) gene. This is a high percentage considering that one cell may contain numerous 16S rRNA copies, which deflates the normalized value.

Levels of *tet*(G) (Figure 1, "*tet*(G)") did not decrease with time after the initial spike of IsoM1-1 into the batch digester, but rather remained approximately 1 to 1.5 log above the background sludge concentration (Figure 1, "*tet*(G) (background)") throughout the experiment. This closely mirrors the maintenance of plate counts at a level also one log above background (Figure 1). It seems likely that the persistence of *tet*(G) at an elevated level is the result of host ARB survival.

However, when *tet*(G) is normalized to 16S rRNA, a decreasing trend can be observed with *tet*(G) ratios returning to background by Day 10. The difference in *tet*(G) and normalized *tet*(G) ratio trends likely arises as a result of 16S rRNA increasing between Day 0 (5.6×10^8 gcn/ml) and Day 10 (1.1×10^{10} gcn/ml) of the study. An increase in 16S rRNA gene copies decreases the normalized ARG ratio. The increase in 16S rRNA concentration could be caused by differential DNA extraction efficiencies between samples or attributed to growth of other digester microbes, possibly in response to the "food" release associated with the death of IsoM1-1, as depicted by plate counts. If this is the case, it appears that the growing microbes did not uptake or otherwise acquire *tet*(G), as shown by the decrease in *tet*(G) ratio (Figure 2).

For comparison, another tetracycline resistance gene, *tet*(W), was measured throughout the experiment. Unlike *tet*(G), *tet*(W) was not detected in the IsoM1-1 pure culture, but was present in the background sludge community. *tet*(W) remained at or near background levels throughout the experiment (Figure 1). The normalized *tet*(G) and *tet*(W) ratios do show a similar trend, albeit the magnitude of the normalized *tet*(W) ratio at the start of the experiment is not as large. As noted previously, the initial high values of normalized *tet*(G) and *tet*(W) ratios are likely in part the result of the relative increase in 16S rRNA values throughout the study. The increase in 16S rRNA would decrease the normalized ratio of any gene that remained consistent, such as the case with *tet*(G) and *tet*(W). Taken in this context, it can be speculated that the growth indicated by 16S rRNA was not a result of ARBs containing *tet*(G) or *tet*(W).

These results are interesting, because it suggests that there is some limit on the maximum concentration of any given species of bacteria within the mesophilic digester, reflecting the

symbiotic balance of a digester microbial community to achieve the necessary hydrolysis, fermentative, and methanogenic reactions. However, the 1-log permanent increase in the concentration of Iso M1-1 reflected in terms of plate count and *tet*(G) concentration suggests that there is room within the mesophilic digester community for a newly introduced microbe (with or without an accompanying ARG) to take root in the community. The time lag in the decline of the *tet*(G) ratio following CFU decline also suggests that some time is required for the digester community to degrade or otherwise re-appropriate the DNA from dead Iso M1-1 cells.

Fate of Tetracycline-Resistant Isolate during Thermophilic Digestion. Iso T10 is a tetracycline-resistant microorganism containing *tet*(W) that was originally isolated from thermophilic (53 °C) digested sludge. As described in the experimental design, a concentrated culture of IsoT10 was spiked into thermophilic digested sludge and incubated at 53 °C for 60-days. Plate counts and ARGs including *tet*(W), which is contained within ARB Iso T10, and *tet*(G), which is present in the background digester community, were monitored over the course of the experiment (Figure 3).

The background plate count of thermophilic sludge on tetracycline-amended tryptic soy agar plates was 7.3×10^2 CFU/ml. The plate count in initial spiked samples was 4.0×10^8 CFU/ml. The plate counts decreased immediately and returned to slightly above background within 15 minutes and remained at approximately 10^3 CFU/ml for 40 days at which point, plating was discontinued.

The *tet*(W) content of the background digester community was 4.3×10^6 gcn/ml (shown as "*tet*(W) background" in Figure 3). Total *tet*(W) content was measured as 2.2×10^6 gcn/ml after addition of concentrated Iso T10 (2.3×10^7 gcn/ml PBS). *tet*(W) levels were lower after the initial spike of Iso T10 into the batch digester perhaps because the pure culture concentration (2.3×10^7 gcn/ml PBS) was so close to the background concentration (4.3×10^6 gcn/ml, Figure 3) that the *tet*(W) increase caused by the spike was small (only 1.4x that of background) and within the QPCR analytical error. It also seems possible that the pure culture of the tetracycline-resistant isolate was not entirely pure or that some members of the population had lost the gene, perhaps as a result of not cultivating the pure culture in broth amended with tetracycline. *tet*(W)

normalized to 16S was 0.03 in the pure culture, suggesting that only 3% of the population contains *tet*(W).

The *tet*(W) concentration increased slightly (0.5 log) within the first 15 days of the experiment, in concert with an increase in 16S rRNA. The increase in *tet*(W) and 16S rRNA could be the result of growth of ARBs (other than Iso T10), some of which harbour *tet*(W). It could also be the byproduct of variable DNA extraction efficiencies, although a similar rise is not observed with background gene *tet*(G). Again, in similar trends, *tet*(W) and 16S rRNA begin a steady decline from Day 15 to the end of the experiment where *tet*(W) and 16S rRNA levels are almost 2 log below background. These results suggest that, as a whole, the biomass fraction was decreasing within the digester after Day 15. Biomass could have been lost as a result of not "feeding" raw sludge to the batch digesters throughout the experiment, where starvation would likely lead to eventual die-off. However, this type of biomass loss was not observed in the mesophilic batch digestion experiments under the same lack of feeding.

For comparison, a background tetracycline resistance gene *tet*(G) was measured throughout the experiment. As shown, *tet*(G) concentrations did not mirror similar trends as *tet*(W) or 16S rRNA, but did decrease relative to background *tet*(G) levels (Figure 3).

Comparison of *tet*(W) and plate counts would yield drastically different decay rates. It doesn't seem likely that a microorganism that was isolated from thermophilically digested sludge would enter a viable but nonculturable state (VBNC) or immediately die upon addition back into the source environment; however, the quick (<15 minute) decline of colony counts from the initial spike to background concentrations would exclude out-competition by other digester microbes. It is possible that the repeated re-streaking events from the original isolation, followed by cultivation in a nutrient-rich broth at 37 °C could have altered the metabolic state or pathways of Iso T10, such that not all members of the Iso T10 community were able to acclimate quickly enough to the immediate re-introduction into the high-temperature, endogenous environment of thermophilic digestion. It is also possible that Iso T10, while isolated from thermophilic digested sludge, was not a viable, active, or continuous member of the thermophilic community, but rather a transient microbe that was present during the initial plating for isolates.

The normalized gene ratios (i.e., ARG gcn normalized to 16S rRNA gcn) for *tet*(W) and *tet*(G) are consistent throughout the experiment (Figure 4). It is unclear as to why the normalized *tet*(G) and *tet*(W) ratios during the experiment are lower than the ratio in the blank sludge sample collected at the start of the experiment. Blank sludge samples were collected from the same sludge used to established the spiked batch digesters and were collected at the same time as time zero experiment samples. Samples were extracted as part of the same batch and analyzed via QPCR on the same well plate.

Lab-scale semi-continuous feed digester study. Concentrations of various ARGs (*sul*1, *tet*(O), and *tet*(W)) and the *int*I1 gene encoding the integrase enzyme of Class 1 integrons were measured in raw sludge feed and mesophilic and thermophilic digester effluents for a period of nine months. This compilation of data depicts similar increasing trends in *sul*1 and *int*I1 (Figures 5a and 5b) concentrations in the raw sludge feed and digester effluents suggesting that the digester communities are subject to the influence of the influent raw sewage feed with respect to *sul*1 and *int*I1. However, *tet*(O) and *tet*(W) levels in the thermophilic digester remain low despite increasing concentrations in the raw sludge feed and mesophilic digester effluent (Figures 5c and 5d).

Discussion

It is worthwhile to consider the possible fate of isolates and the ARGs contained within the isolate host cell. An increase in ARG content of the digester could occur as a result of differential survival and growth of the host cell, horizontal gene transfer from the host cell to other microorganisms of the digester community, lengthy persistence of extracellular ARGs (from lysed host cells), or a combination thereof. A decrease in the ARG content of the digester could occur as a result of death, lysis, and degradation of the host cell and DNA contents or expulsion and subsequent degradation of the extracellular ARG mobile genetic element.

The maintenance of *tet*(G) concentrations at initial elevated spike levels combined with a substantial decrease in the CFU plate counts of Iso M1-1 suggest that horizontal gene transfer did occur in the mesophilic batch digestion experiment. Although normalized *tet*(G) ratios show a decrease to background levels as a result of 16S rRNA increasing throughout the experiment, it may be more telling to evaluate the trend of *tet*(G) to CFU plate counts. Stability in this ratio

would indicate that *tet*(G) was perishing at the same rate as the host ARB. Persistent *tet*(G) concentrations while CFU plate counts decrease, as shown in this study, would suggest the transfer of *tet*(G) to the digester community. Merlin et al. (2011) observed similar loss of donor bacteria DNA (laboratory strain E. coli DH5α containing plasmid pB10) with a concurrent relative stability of plasmid pB10 DNA in batch mesophilic (35 °C) anaerobic digestion experiments and concluded that the ARG plasmid had entered the indigenous population via conjugative transfer. However, CFU levels did not decrease to background levels, suggesting that a portion of the spiked Iso M1-1 bacteria survived. These results differ from the thermophilic study where plate counts returned to background concentrations. This suggests the survival of a higher proportion of introduced tetracycline resistant bacteria in mesophilic batch experiments relative to thermophilic batch experiments. Survival and in particular, growth, of ARBs introduced into any treatment environment represent a reservoir of donor bacteria for future transfer and a reservoir of available ARGs for dissemination (Mao et al., 2014).

In thermophilic batch digestion experiments with Iso T10 harboring *tet*(W), the loss of CFU plate counts coupled with the eventual decline in *tet*(W) concentrations below background levels suggest that with this particular microorganism, horizontal gene transfer did not occur. Merlin et al (2011) observed the simultaneous loss of donor and plasmid DNA in activated sludge experiments, concluding that the ARG plasmid perished with the host. The data from this study are less conclusive because the decline in *tet*(W) concentration was accompanied by a decline in 16S rRNA and did not occur at the same rate as the decline in CFU plate counts. These results may indicate that *tet*(W) was quickly transferred, then declined as the population as a whole declined (as represented by the 16S rRNA). Alternately, the *tet*(W) from dead donor cells may have persisted as extracellular DNA or within dead unlysed cells and degraded at a slower rate than host death. Other experiments (Chapter 2) with methicillin-resistant *Staphylococcus aureus* (MRSA) and *E. coli* fate in mesophilic digestion have shown the persistence of both donor chromosomal and plasmid DNA approximately 10 days beyond the non-detection of plate counts. Similar studies in thermophilic digestion have shown the persistence of both donor chromosomal and plasmid DNA longer than 60 days, although the presence of viable but nonculturable bacteria (VBNC) bacteria could not be discounted. However, the large background concentration of *tet*(W) could have overwhelmed any effect that may have been

exerted by the addition and subsequent death of Iso T10. Also, differentiating between these mechanisms of ARG attenuation or persistence is not possible at CFU concentrations at or below background levels because a microbe-specific gene was not identified and it was not possible to distinguish Iso T10 colonies from other background tetracycline-resistant bacteria at these dilution levels.

The batch digester results suggest that the ARG content of a digester cannot be separated from the fate of the ARG host, in particular in mesophilic digestion where a wider variety of microorganisms are able to assimilate, adjust, survive, or even thrive in the digester conditions. It seems likely that the reason the tetracycline ARG content in thermophilic digestion is less variable than mesophilic digestion is the more narrow diversity of microbes that are able to inhabit the thermophilic environment. In addition, these experiments have shown horizontal gene transfer likely occurs in mesophilic digestion, although the results are not as clear in thermophilic digestion.

The ability of a newly introduced species (possibly containing an ARG) to survive, grow, and occupy a niche within the mesophilic digester community is also supported by the continuous feed experiments. Here, the rising trend in raw sludge ARG concentration of *int*I1 and *sul*1 is mirrored by a rising trend in the *int*I1 and *sul*1 ARG concentration in both the mesophilic and thermophilic digester effluents (Figures 5a and 5b). The rise in *int*I1 and *sul*1 concentrations within the continuous feed digesters concurrent with the rise in raw sludge ARG could be explained by the survival or selective enrichment of influent raw sludge ARBs containing *int*I1 or *sul*1 or by horizontal gene transfer between incoming raw sludge bacteria and the digester community.

There was no clear trend in *tet*(O) and *tet*(W) concentrations in raw sludge or mesophilic digester effluent (Figures 5c and 5d); however, the *tet*(O) and *tet*(W) concentrations in the thermophilic digester remained consistently low over the entire monitoring period. These results suggest that fates of incoming raw sludge ARBs containing *tet*(O) and *tet*(W) or the rates of horizontal gene transfer were variable within the mesophilic digester, while the thermophilic digester community was relatively immune to ARG intrusion.

The variable response of different ARGs within the same digester may not be a surprise, considering *int*I1 is the integrase gene associated with the highly mobile Class 1 integrons and *sul*1 is often co-located on Class 1 integrons (Paulsen, et al., 1993; Zhang et al., 2009). *tet*(W) and *tet*(O) are found on plasmid and chromosomes, but not necessarily associated with integrons. Thermophilic digestion may be able to achieve higher ARG content removal with respect to tetracycline genes because of the limited occurrence of *tet* genes within this particular thermophilic digester community, the incompatibility of the incoming raw sludge bacteria with the thermophilic digestion community for horizontal gene transfer, and the harsh environment restricting entrance into the digester community of new ARBs containing *tet* genes. These results also suggest that limiting the ARG content of the initial seed community of a thermophilic digester may help limit the ARG content of digester effluent - i.e., biosolids intended for land application. Again, these results highlight the importance of not only horizontal gene transfer, but also host survival. The limited diversity of a thermophilic digester (Wilson and Novak, 2008) and limiting (high temperature, high ammonia) conditions of a thermophilic digester likely limit opportunities for horizontal gene transfer and limit survival of incoming ARBs.

Conclusions

The presence of a very diverse bacterial, mobile genetic element, and ARG population in activated sludge systems has recently been highlighted by metagenomic studies (Zhang et al., 2011). The activated sludge environment is characterized by ambient temperatures (10 to 25 ºC), rich nutrient and organic loading, dense microbial populations. Similar conditions also exist in mesophilic digestion, albeit with a higher and more closely controlled temperature regime (~37 ºC). In contrast, thermophilic regimes operate at high temperatures, endogenous conditions, with very high ammonia concentrations. Thermophilic digestion environments have been shown to support a less diverse microbial population than mesophilic digestion (Wilson and Novak, 2008). Moreover, to gain critical time-temperature requirements for production of Class A biosolids classification, thermophilic digesters are operated with minimum retention times prior to effluent withdrawal and shorter SRTs. This may allow longer times of uninterrupted degradation between raw sludge additions and shorter solids retention times for HGT to occur. These environmental and operating conditions may give thermophilic digestion a competitive advantage over activated sludge or mesophilic digestion in the reduction of ARG content of biosolids. Moreover, although

HGT continues to be an important mechanism of ARG persistence for some highly mobile ARGs (*sul*1, *int*I1), it appears that thermophilic digestion may allow for the reduction of other ARGs ((*tet*(O), *tet*(W)) through incoming ARB death and narrowed host range for HGT.

It is lofty to entertain the idea that seeding a thermophilic digester with ARG-free microorganisms could lead to an ARG-free effluent (biosolid). It seems plausible that due to the apparent limitations on microbial diversity and the resultant limitations on HGT, that thermophilic digestion could achieve a higher rate of ARG reductions compared to raw sludge. However, it is evident that HGT still does occur, particularly with highly mobile integrons, so that complete reduction of all ARGs would not be possible with thermophilic digestion alone. Further investigation into pre-treatments designed for complete cell lysis and possibly DNA degradation in raw sludge are warranted.

In conclusion, HGT is important more so in mesophilic digestion than thermophilic digestion because of the diverse microbial population and broad host range. ARB survival and growth is an important factor in ARG fate in both digestion regimes.

Acknowledgements

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Figures

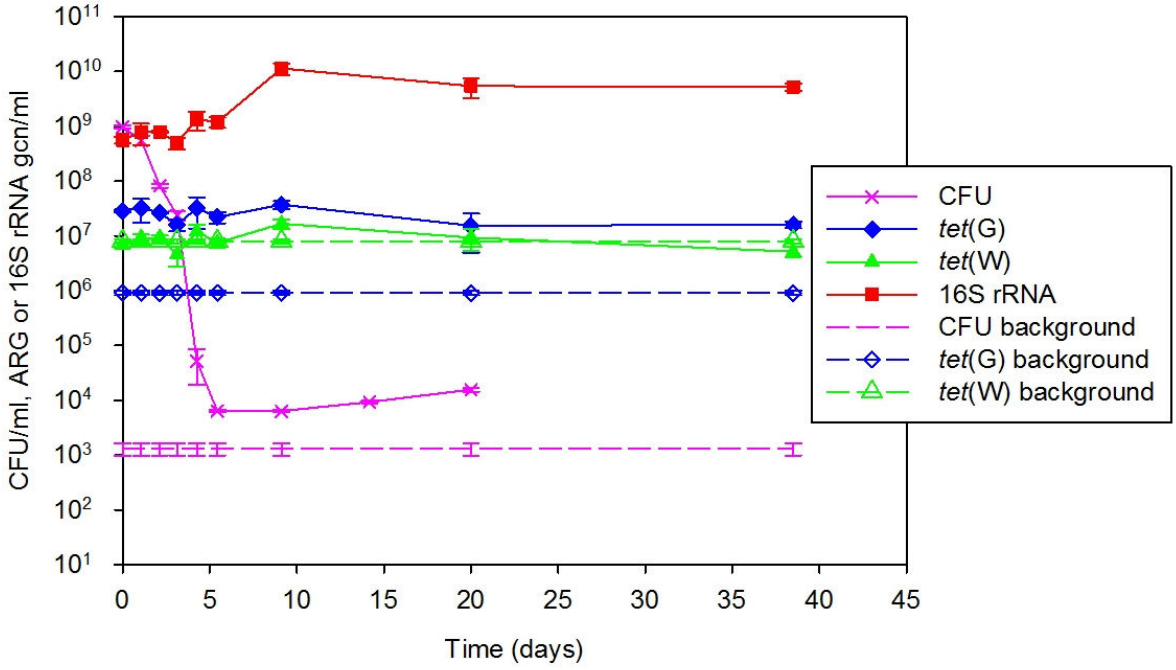

Figure 3-1. Plate count (CFU/ml sludge) and ARG and 16S rRNA (gcn/ml sludge) for *tet*(G) associated with tetracycline-resistant isolate M1-1 and *tet*(W) in the background community at 37 °C in digested sludge.

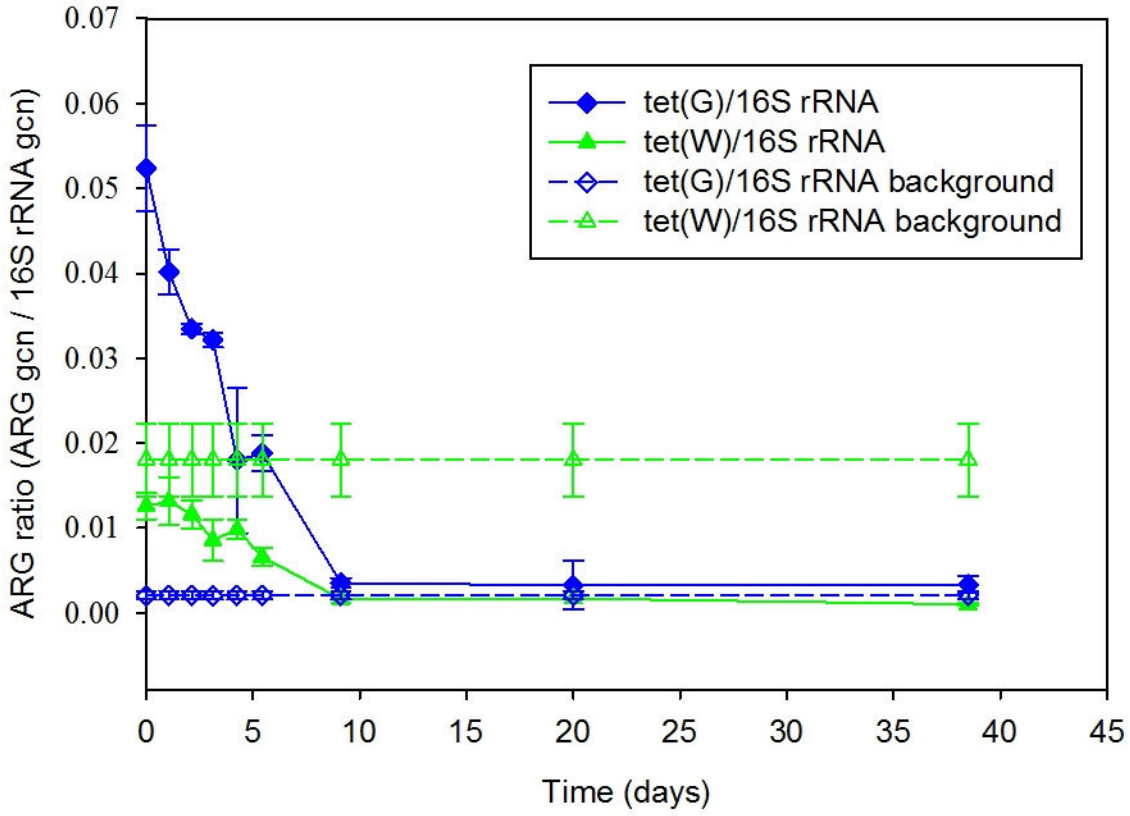

Figure 3-2. Normalized ARG ratios (ARG gcn / 16S rRNA) for *tet*(G) associated with tetracycline-resistant isolate M1-1 and *tet*(W) in the background community at 37 °C in digested sludge. Background line represents the pre-spike concentration in digested sludge.

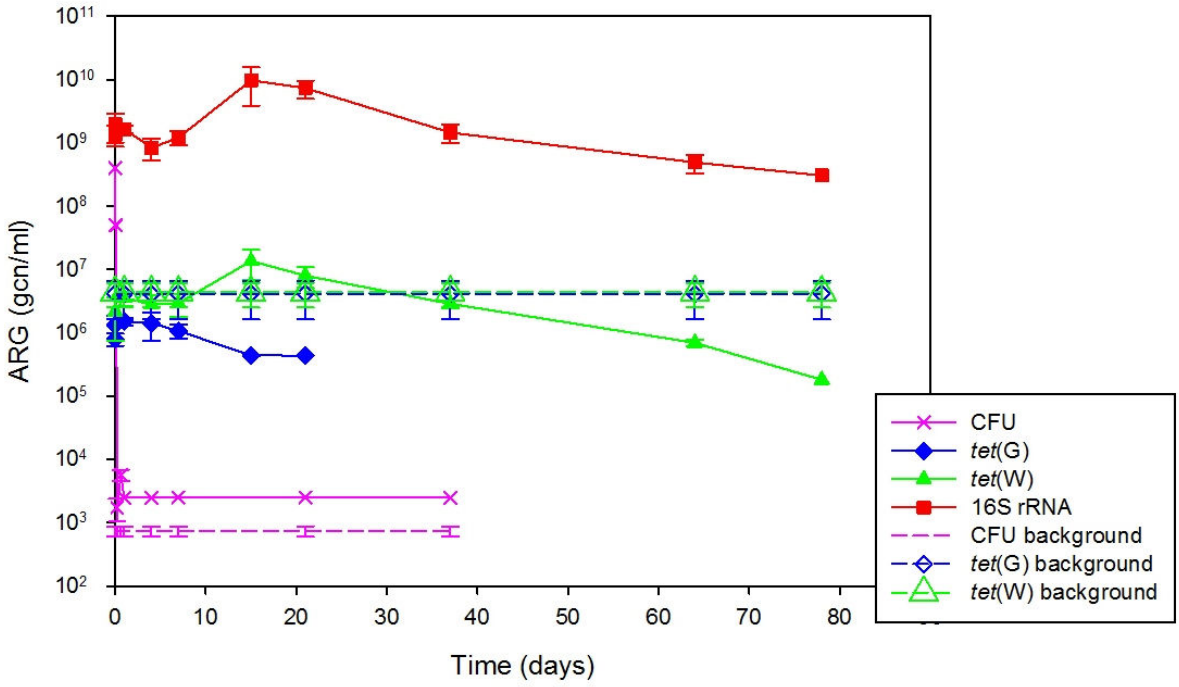

Figure 3-3. Plate count (CFU/ml) and ARG and 16S rRNA (gcn/ml sludge) for *tet*(W) associated with tetracycline-resistant isolate T10 and *tet*(G) in the background community at 53 °C in digested sludge.

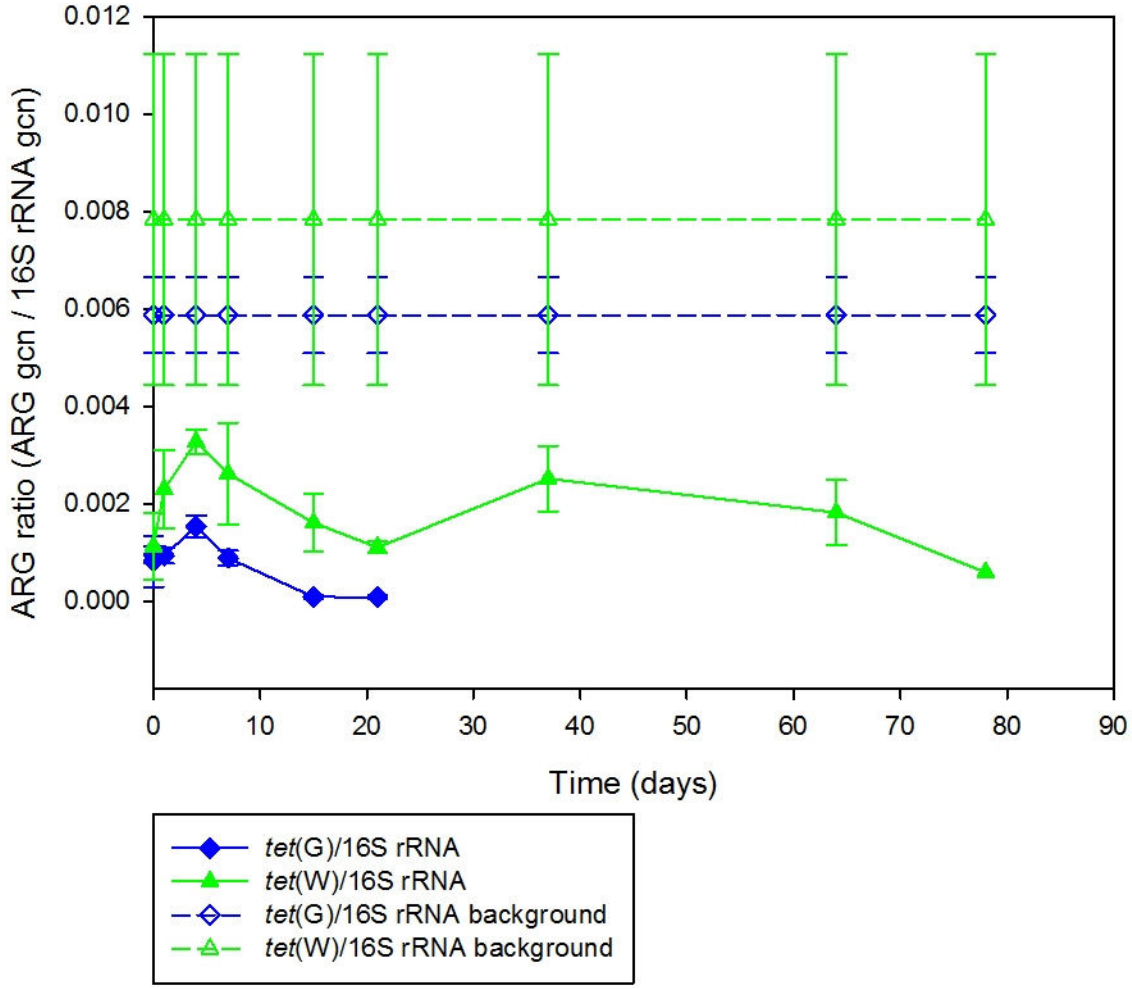

Figure 3-4. Normalized ARG ratios (ARG gcn / 16S rRNA) for *tet*(W) associated with tetracycline-resistant isolate T10 and *tet*(G) in the background community at 53 °C in digested sludge. Background line represents the pre-spike concentration in digested sludge.

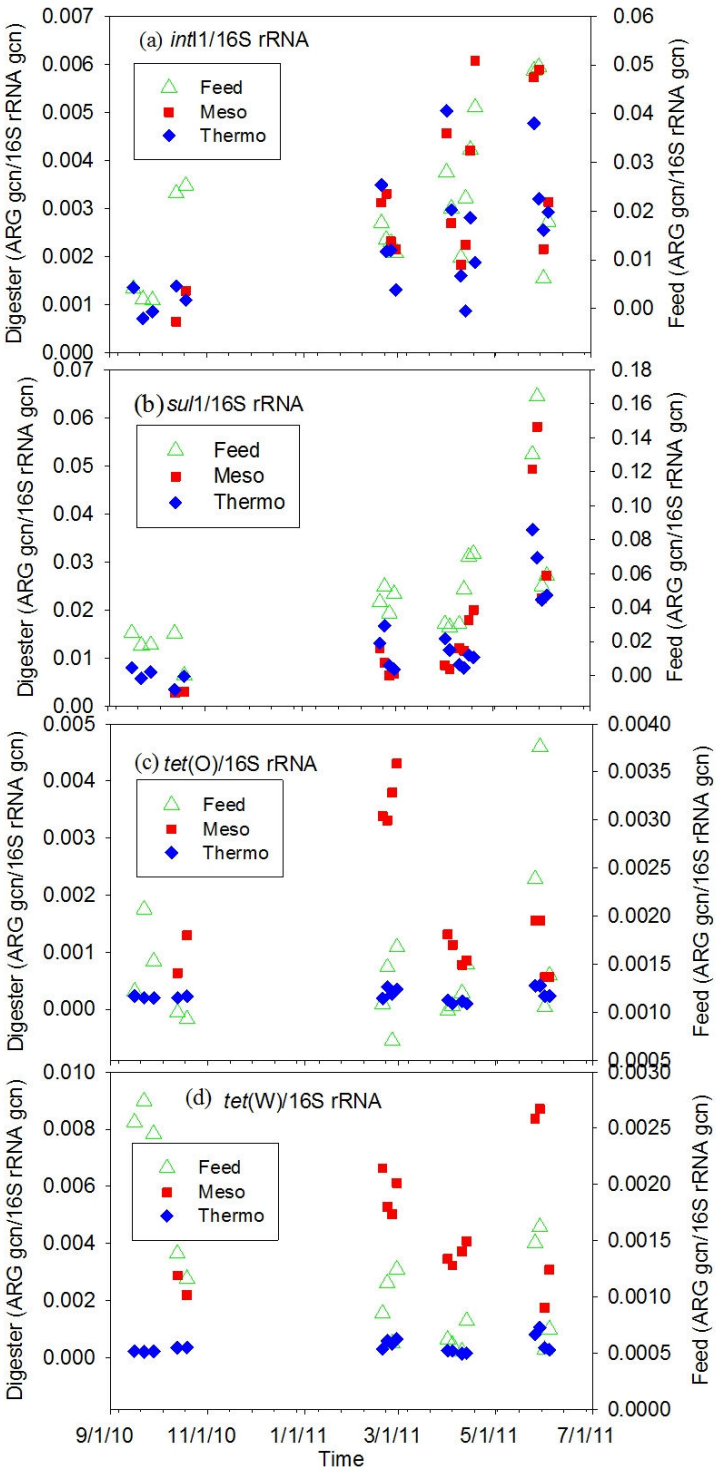

Figure 3-5. ARG concentrations including *int*I1 (Figure 5a), *sul*1 (Figure 5b), *tet*(O) (Figure 5c), and *tet*(W) (Figure 5d) in raw sludge, mesophilic digester effluent, and thermophilic digester effluent during nine months of monitoring of semi-continuously fed lab-scale digesters.

CHAPTER 4

Effect of Ag Nanoparticles and Antibiotics on Antibiotic Resistance Genes in Anaerobic Digestion

Jennifer H. Miller, John T. Novak, William R. Knocke, Katherine Young, Yanjuan Hong, Peter J. Vikesland, Matthew S. Hull, Amy Pruden

Abstract

Wastewater treatment plants have been described to create "breeding ground" conditions for the selection, transfer, and dissemination of antibiotic resistance genes (ARGs) among various bacteria. The objective of this study was to determine the effect of direct addition of antibiotic and Ag NPs on the occurrence of ARGs in thermophilic anaerobic digesters. Test thermophilic digesters were amended with environmentally-relevant Ag NP (0.01, 0.1, and 1.0 mg-Ag/L; corresponding to ≈0.7, 7.0, and 70 mg-Ag/kg total solids (TS)) and sulfamethoxazole (SMX) concentrations that span susceptible to resistant classifications (1, 5, and 50 mg/L) as potential selection pressures for ARGs. Tetracycline (*tet*(O), *tet*(W)) and sulfonamide (*sulI*, *sulII*) ARGs and the integrase enzyme gene (*intI1*) associated with class 1 integrons were measured in the raw sludge, test thermophilic digesters, a control thermophilic digester, and a control mesophilic digester. There was no apparent impact of Ag NPs on thermophilic anaerobic digester performance. The maximum SMX level (50 mg/L) resulted in accumulation of volatile fatty acids (VFAs) and low pH, alkalinity, and volatile solids reduction (VSR). There was no significant difference between ARG gene copy numbers (absolute or normalized to 16S rRNA genes) in amended thermophilic digesters and the control thermophilic digester. ARG gene copy numbers in digested sludge ranged from 10^3 to 10^6 copies per microliter (approximately $8x10^1$ to $8x10^4$ copies per microgram) of sludge as result of one log reduction of ARGs (two log reduction for *intI1*). Quantities of the five ARGs in the raw sludge ranged from 10^4 to 10^8 copies per microliter (approximately $4x10^2$ to $4x10^6$ per microgram) of sludge. Test and control thermophilic digesters (53 °C, 12-day solids retention time (SRT)) consistently reduced, but did not eliminate, levels of all genes that were analyzed. The mesophilic digester (37 °C, 20-day SRT) also reduced levels of *sul*I, *sul*II, and *int*I1 genes, but levels of *tet*(O) and *tet*(W) were the same or higher than in raw sludge. The fact that ARG reductions remained constant in spite of

the application of selection pressures suggest that the digester operating conditions themselves are a strong factor governing the composition of the bacterial community and thus the prevalence of ARGs.

Keywords: Silver nanoparticles; nanosilver; Ag NPs; Ag; ionic silver; thermophilic anaerobic digestion; mesophilic anaerobic digestion; antibiotic resistance genes; ARGs; antibiotic; sulfamethoxazole

Introduction

Wastewater treatment plants (WWTPs) have been identified as vehicles for the conveyance of pharmaceuticals and silver nanoparticles (Ag NPs, or nanosilver) as well as antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) from anthropogenic sources to the environment. Kolpin et al. (2002) raised the awareness of pharmaceuticals (including antibiotics) in the environment, sourced in part from WWTPs. The list of concerns has now grown to include engineered nanomaterials, such as Ag NPs, which are used primarily as antimicrobial agents in consumer products. The Project on Emerging Nanotechnologies reports that as of March 2011, Ag NPs are used in 24% of the 1300 identified commercially available, nanotechnology-enabled products. Benn and Westerhoff (2008) reported that 10 to 30% of silver contained in a tested Ag NPs-amended sock was released to washwater as Ag NPs. This percentage and overall silver mass varied widely by manufacturer and sock type, but the findings underscore the anticipated increase of Ag NPs loading to wastewater treatment plants. Finally, antibiotic resistance genes (ARGs) are also emerging contaminants of concern (Pruden et al. 2006).

ARGs genetically encode a molecular mechanism, such as efflux pumps, target modification, or antibiotic inactivation or degradation, that enables bacteria to circumvent or diffuse antibiotic action. Many infectious diseases are increasingly difficult to treat because of antimicrobial-resistant pathogenic organisms (National Institute of Allergy and Infectious Diseases, 2011). Pathogenic bacteria differ little from commensal or environmental bacteria in the basic cell functions that are the target of antibiotics; thus genetic exchange of mobile resistance elements can and does occur in the environment and not just in clinical settings (Summers, 2002). WWTPs with high nutrient loadings and dense microbial populations have been identified as potential breeding grounds for amplification and transfer of ARGs (Kim and Aga, 2007).

An overarching hypothesis of this study is that antibiotics and Ag NPs could act as selection agents for ARG proliferation in anaerobic digesters. Kohanski et al. (2010) reported that sub-lethal antibiotic concentrations produced an oxidative stress response, which induced mutagenesis and thereby resulted in an increase in resistance to multiple antibiotics. In nature, antibiotics at low concentrations may act as signals that initiate transcription of genes to alter metabolism and growth (Allen et al., 2010; Davies et al., 2006) suggesting that antibiotics have biological consequences even at sub-inhibitory concentrations.

Co-selection of antibiotic and silver resistance genes has recently been documented (Cunningham and Lin, 2010; Loh et al., 2009). Genes encoding silver resistance and antibiotic resistance can be found on the same plasmid or in the same bacteria on different plasmids and/or other loci. Integrons can also facilitate co-selection. Integrons are multi-gene cassettes that can encode many different ARGs under a common promoter. Thus, selection pressure exerted by one antibiotic may select for ARGs corresponding to distinct antibiotics within the gene cassette of the integron. For example, *sul*I is an ARG that incurs resistance against sulfonamides, including sulfamethoxazole (SMX), and it is located in the conservative region of Class I integrons. Persistent SMX could co-select for ARGs encoding resistance to other clinically important antibiotics that are co-located on integrons.

To date, three published studies have examined the effect of anaerobic digestion on ARGs (Ghosh et al., 2009; Diehl and LaPara, 2010; Ma et al., 2011). Together, full-scale and lab-scale studies measured ARG numbers and ratios (ARG gene copy number normalized to 16S bacterial rRNA) for tetracycline, sulfonamide, erythromycin, and Class 1 integrase genes in thermophilic and mesophilic digestion. All genes showed some reduction during thermophilic treatment; however, results for tetracycline genes in mesophilic digestion have varied, suggesting that the digester microbial community and the operating conditions that influence the development and maintenance of that community play an important role in determining ARG fate. Aside from temperature, digester operating conditions and raw sludge characteristics (such as ARG composition or antibiotic and Ag NPs concentration) that may affect digester ARG composition have not been investigated.

The fate and toxicity of ionic silver in wastewater processes has been previously reported (Lytle, 1984; Bard et al., 1976; Pavlostatis and Maeng, 2000; Shafer et al., 1998). However, concerns have arisen as to whether Ag NPs pose hazards distinct from ionic silver with respect to wastewater microbes, toxicity, and fate (Liang et al., 2010; Choi and Hu, 2009). Kiser et al. (2010) reported a 97% removal of Ag NPs by sorption to activated sludge biomass; thus, Ag NPs will likely accumulate from influent wastewater in sludge sent to digestion. Yang et al. (2012) reported that an Ag NP concentration of <40 mg/L had a negligible impact on anaerobic digestion as assessed by methane production, pH, volatile fatty acids (VFAs), and methanogen diversity and population.

With respect to SMX, Fountoulakis et al. (2004) did not measure any inhibition of acetoclastic methanogenesis under mesophilic conditions by SMX at concentrations up to 400 mg/L as measured by specific methanogenic activity. The impacts of SMX on thermophilic anaerobic digestion processes also have not been previously reported.

The purpose of this study was to investigate the fate of ARGs in thermophilic anaerobic digestion, the impact of artificially-applied antibiotic (SMX) and antimicrobial (Ag NPs) selection pressures on ARGs and anaerobic digester performance, and possible co-selection of multiple ARGs via integrons. SMX, citrate-coated Ag NPs, and ionic silver were spiked separately at increasing concentration levels into raw sludge and fed daily to lab-scale digesters. Anaerobic mesophilic and thermophilic control digesters (no amendments) were also operated for comparison. Silver levels were selected to mirror estimated environmentally relevant concentrations of Ag NPs ranging from 0.01 to 1 mg-Ag/L (Benn and Westerhoff, 2008; Gottschalk et al., 2009; Muhling et al., 2009; USEPA, 2009). rather than minimum inhibitory values (MIC), which have been reported to range from 0.02 to 65 mg/L in susceptible bacteria and up to 2675 mg/L in resistant bacteria (Clement and Jarrett, 1994). Bard et al. (1976) found silver nitrate concentrations of 10 mg-Ag/L inhibited wastewater bacteria. SMX concentrations (1, 5, and 50 mg/L) were selected based on background SMX concentrations in biosolids (USEPA, 2009) and MIC values corresponding to susceptible (<2 to 38 mg/L) and resistant (>8 to 150 mg/L) as reported in Nagulapaly et al. (2009). Background SMX concentrations in wastewater have been reported in the range of 0.1 to 5 µg/L and in biosolids in the range of 3.91 to 651 µg/kg (0.05 to 7.8 mg/L assuming 1.2% TS, USEPA, 2009). Bacterial 16S rRNA genes

and ARGs encoding resistance to sulfonamides (*sul*I, *sul*II) and tetracycline (*tet*(O), *tet*(W)) as well as a gene encoding the integrase enzyme (*int*I1) that is found in Class 1 integrons were measured in the raw sludge feed and digested sludge (i.e., bioreactor effluent). Class 1 integrons are the largest, most prevalent integron class and contain most integron-associated ARGs. *sul*I is located in the conservative region of Class I integrons and encodes resistance to sulfonamides including SMX. ARGs *tet*(O) and *tet*(W), which enable resistance to tetracycline by protecting ribosomes, were targeted because they have been shown to be associated with wastewater sources (Storteboom et al., 2010) and have shown variable treatment efficiencies within mesophilic digestion (Diehl and LaPara, 2010; Ma et a., 2011).

Methodology

***Digester operation*.** A total of four 15 L, 12-day solids retention time (SRT) thermophilic (53°C) digesters and one 10 L, 20-day SRT mesophilic (37°C) digester were fed raw sludge daily. Three of the thermophilic digesters were used as test digesters and received feed with either Ag NPs, silver nitrate, or SMX at three concentration levels. One thermophilic control digester and one mesophilic control digester with no amendments were also included.

High density polyethylene cone fermenters (Hobby Beverage Equipment Company, Temecula, California) with nominal volumes of 6.5 gallons (24.6 liters) were fed daily with a 70% primary sludge and 30% thickened waste activated sludge mixture from the Christiansburg, Virginia WWTP, which predominantly receives residential wastewater with minimal industrial contribution and no hospital contributions. This mixture was diluted with tap water to 2.5% total solids (TS) to maintain a consistent feed to the digesters to reduce variations in operating parameters (e.g., pH, alkalinity, VFAs, volatile solids reduction (VSR)). During this study, raw sludge was collected from the plant every four to six weeks and stored at 4°C until fed into the digesters. Total solids, volatile solids (VS), and pH of the raw sludge were routinely measured to ensure consistency of the feed sludge over the study period. A peristaltic pump was used to mix digester contents by recycling headspace gas to the bottom of the cone digester. Evolved gases were collected in 25-liter Tedlar bags (SKC, Inc., Eighty Four, Pennsylvania). All digesters were maintained in a 37°C constant temperature room.

The mesophilic anaerobic digester was operated at an active volume of 10 L (2.6 gallons), 20-day SRT, with heating to 37°C achieved from ambient conditions. The digester was initiated using 5L of digested sludge seed from the Christiansburg WWTP. One-half L of feed sludge mixture was added per day until the 10L operating volume was reached. The four thermophilic anaerobic digesters were each operated at an active volume of 15L (4 gallons), 12-day SRT, and heated to a target temperature of 53°C using electric heating tape with a temperature controller (Model No. BSAT 101-100, Thermolyne, Dubuque, Iowa). The temperatures (°C) in the thermophilic digesters over the course of the study were: Ag NPs, 52.0 ± 1.3; Ionic silver, 52.7 ± 1.3; SMX, 52.2 ± 1.8; Thermo control, 53.2 ± 1.7. These digesters were seeded with digested sludge from laboratory thermophilic anaerobic digesters (temperatures ranging from 48°C to 57°C) fed with primary and secondary solids from District of Columbia Water and Sewer Authority Blue Plains Advanced Wastewater Treatment Plant. The start-up of mesophilic and thermophilic digesters took place over a 6-month period prior to these studies and the studies were conducted for another six months.

Digester Performance Monitoring. Total and volatile solids (Method 2540-G), total alkalinity (Method 2320-G), pH (Method 4500), and gas volume (Method 2720) were analyzed as specified in Standard Methods for the Examination of Water and Wastewater (APHA, 1995). Headspace methane and carbon dioxide were analyzed on a Shimadzu Gas Chromatograph (Model GC-14A) with a thermal conductivity detector (TCD) as outlined in Wilson et al. (2008). VFA were measured using a Shimadzu gas chromatograph (Model GC-14A) with flame ionization detector (FID) as outlined in Wilson et al. (2008). Individual acid concentrations are converted to acetate on a theoretical oxygen demand basis and summed to report Total VFA as mg/L as acetate.

Total silver was measured in digested sludge from each digester prior to the start of studies to establish a baseline. These values ranged from 0.9 to 9.6 mg Ag/L. These values are higher than values reported by the WWTP, where annual total silver concentration in biosolids from 2009 to 2011 were reported as <5.6 mg/kg, 22 mg/kg, and <14.9 mg/kg (equivalent to 0.07, 0.19, and 0.26 mg Ag/L, assuming 1.2% TS).

Dosing of selection pressures. Following start-up, all digesters received raw sludge without any amendments to establish a baseline and to ascertain typical variation of ARG concentrations between digesters that are fed the same raw sludge. Digester performances were monitored and samples were analyzed by quantitative polymerase chain reaction (QPCR) over 3 SRTs. ARG ratios and un-normalized ARGs were the same ($p<0.05$) in digested sludge from all thermophilic digesters during this baseline time period (data not shown).

Following the baseline, test digesters were amended with either Ag NPs, ionic silver, or SMX at three sequential concentration levels including Level 1 (0.01 mg Ag/L AgNPs, 0.01 mg Ag/L ionic Ag, or 1 mg/L SMX), Level 2 (0.1 mg Ag/L AgNPs, 0.1 mg Ag/L ionic Ag, or 5 mg/L SMX), and Level 3 (1.0 mg Ag/L AgNPs, 1.0 mg Ag/L ionic Ag, or 50 mg/L SMX). On Day 0 of each concentration level, the appropriate volume of stock solution (or deionized (DI) water for control digesters) was added to a given digester to achieve 25% of the target concentration. Thereafter, the daily feed mixture was amended with Ag NPs, ionic silver, SMX, or DI water. This process was intended to avoid any shock loading. It was determined that 90% of the target concentration would be reached within 2 SRTs. After 2 SRTs (starting Day 25), low variation in VSR and pH (standard deviation <10% or less of average value) were used to verify steady state conditions and digester performance and QPCR samples were collected for an additional 1.5 SRTs thereafter.

Ag NPs synthesis. Ag NPs were synthesized using the method modified from Solomon et al. (2007) with stabilization by sodium citrate (2mM). The final silver concentration was 10.4 mg-Ag/L as measured by inductively coupled plasma mass spectrometer. UV-Vis spectroscopy measurements were obtained using a Cary 5000 UV-Vis-NIR spectrophotometer (Agilent Technologies). Estimates of hydrodynamic diameter (Z-average, 44.6 ± 3.9 nm) were obtained using a Malvern Zetasizer NanoZS (Malvern, UK). The polydispersity index ranged from 0.524 to 0.885 suggesting that suspensions were similarly monodisperse.

Ionic silver preparation. Silver nitrate (Sigma Aldrich, St. Louis, Missouri) was dissolved in distilled water to a concentration of 10.4 mg-Ag/L.

SMX preparation. SMX (Sigma Aldrich, St. Louis, Missouri) was dissolved in methanol with dilution by distilled water to a final concentration of 500 mg/L and 100 mg/L.

Quantification of ARGs. DNA was extracted from 250 µL of digested sludge from the digesters or raw feed sludge (250 mg centrifuged sludge for Baseline samples) using FastDNA Soil Kit (MP Biomedicals, Solon, Ohio) according to manufacturer's protocol. Extracted DNA was diluted 50× to minimize inhibitory effects (Ma et al., 2011). Diluted DNA extracts were stored at -20°C until analysis by QPCR for bacterial 16S rRNA genes, *sul*I, *sul*II, *tet*(O), *tet*(W), and *int*I1. A 10 µL reaction mixture was comprised of 5.0 µL SsoFast Evagreen Supermix (Bio-Rad, Hercules, California), 0.8 µL of each 5 µM primer (Ma et al., 2011), 2.4 µl molecular biology grade water, and 1 µL of DNA template. All samples were quantified in triplicate. Standards prepared from serial dilutions of cloned genes ranged from 10^2 to 10^8 gene copies per µL. Standards and a reagent blank were included in each run.

Quantification of methanogen populations. Diluted DNA extracts were analyzed by QPCR using TaqMan® probes and primers targeting for order-specific 16S rRNA archael genes corresponding to hydrogenotrophic methanogens (*Methanobacteriales* (MBT), *Methanococcales* (MCC), and *Methanomicrobiales* (MMB) and an acetoclastic methanogen *Methanosarcinales* (MSL) (Wilson, 2009).

Statistics. Microsoft Excel 2007 was used to calculate averages and standard error of all data. R 2.8.1 (http://cran.r-project.org/bin/windows/base/old/2.8.1) was used to perform t tests on log ARG gene copy numbers and ARG ratios between 1) test digesters and the thermophilic control digester and 2) all digesters and the raw sludge feed. A p-value of <0.05 was considered to indicate a significant difference between means.

RESULTS

Digester Performance. There was no observed effect of environmentally-relevant concentrations of Ag NPs or ionic silver compounds on thermophilic digester performance. Also, there was no observed effect of SMX at concentrations below the MIC (1 and 5 mg-SMX/L). The pH was maintained between 7.4 and 7.6 with alkalinities between 3,200 and 4,100 mg/L as $CaCO_3$. VSR ranged between 54 to 62% and total VFAs ranged between 64 and 180 mg-acetate/L with acetate and propionate comprising almost all VFAs. Statistical analyses of pH, methane gas composition, gas volume, and VSR data showed that all thermophilic test digesters operated comparably to the thermophilic control digester when fed the same raw sludge except a slightly

lower VSR in the ionic Ag digester during Level 3 ($p<0.05$, Figure 1). The mesophilic control digester operated at a lower pH and produced less gas, which is to be expected given the difference in daily feed volumes. The methane composition of the mesophilic control digester was lower during Level 1 than the thermophilic control because the digester had a small air leak, but this did not appear to affect VSR in Level 1. Level 3 VSR in the mesophilic control was lower than the thermophilic control. This deviation was likely caused by the continued improvement in VSR and stability with digester age in the thermophilic digesters, which tend to require longer acclimation periods, rather than deterioration in the performance of the mesophilic digester. The mesophilic digester VSR performance was consistent across all dosing levels.

Digester performance was adversely affected by the high dose of SMX (50 mg/L). Alkalinity decreased to 2,260 mg/L as $CaCO_3$ and the pH decreased to 6.3 in response to an increase in VFAs. The VSR declined to 40%, at which point SMX dosing was suspended. Gas composition was difficult to interpret because total gas recoveries (i.e., CO_2 plus CH_4) increased from 103% up to as much as 114% during this period. This could be explained by TCD detection of other un-identified components in the CO_2 and CH_4 fractions.

Interestingly, there was a 30% increase in gas volume production in the first few days of SMX addition at 50 mg/L, followed by a return to the baseline volume production (data not shown). Hydrogen gas concentrations were two to six times the concentration of the thermophilic control digester (data not shown). The color of the digested sludge turned from black to a light brown suggesting that digestion was not complete (also supported by VSR measurements), while the odor changed from earthy-musty to acidic or vinegar (acetic acid).

QPCR analysis of sludge samples collected from the SMX digester show that hydrogenotrophic and acetoclastic methanogen populations measured both as gene copy numbers per microliter and gene copy numbers per gram volatile solids were reduced at the time coinciding with reductions in VSR (data not shown). The relative proportion of hydrogenotrophic and acetoclastic methanogens did not change (data not shown).

Effect of Ag NPs and SMX on ARGs. The results of QPCR analysis are provided as ARG copy numbers normalized to 16S bacterial rRNA (ARG ratio) as well as ARG copy number per microliter of sludge (un-normalized ARGs). Normalization accounts for variability in DNA

extraction efficiencies and biomass between samples. ARG ratios (Figure 2) can be thought of as roughly representing an average gene count per bacteria cell and as such, can mask increases or decreases in un-normalized ARG copy numbers as a result of biomass growth or death. For this reason, un-normalized ARG data are also provided (Figure 3).

There was no consistent trend of ARG copy numbers or ratios with respect to dosing concentration for any of the digesters. ARG ratios in test digesters (Ag NPs, ionic silver, SMX) were the same as the thermophilic control digester ($p > 0.05$) for all genes during all sampling periods except for *sul*I and *sul*II ($p < 0.05$) were lower in the Ag NPs and SMX digesters at Level 2 (0.1 mg-Ag/L and 5 mg-SMX/L, respectively) (Figure 2, "*" symbol). Analysis using un-normalized ARGs (Figure 3, "*" symbol) also showed the same result of test digesters predominantly being the same as the thermophilic control digester; however, the exceptions were *tet*(O) in the ionic silver digester at Level 1 (0.01 mg-Ag/L), *sul*II in the Ag NP and SMX digesters at Level 2 (0.1 mg-Ag/L and 5 mg-SMX/L, respectively), and *tet*(O) in the Ag NP digester at Level 3 (1.0 mg-Ag/L).

Effect of digester temperature on ARGs. In comparing digested sludge to raw sludge feed (Figures 2, "#" symbol), the ARG ratios in the test digesters and the thermophilic control digester were different (lower) from the raw sludge feed ($p<0.05$), except *int*I1, *sul*I and *tet*(W) in all digesters during Level 3 and *tet*(O) in the ionic silver and AgNP digesters during Level 3. However, the p values of these exceptions were small ($p<0.09$) and were the result of the high variability of the feed ARG ratios stemming from the storage conditions. Statistical analysis using un-normalized gene copy (Figure 3, "#" symbol) showed all ARGs in thermophilic digesters were lower than the raw sludge feed. The un-normalized gene data also shows that the 16S rRNA gene is lower in AgNP and ionic silver digesters during Level 2 and Level 3 and the SMX digester during Level 2.

tet(O) and *tet*(W) ratios in the mesophilic control digester were the same or higher from the raw sludge feed for all sampling periods. Similar to the thermophilic digesters, *int*I1 and *sul*I ratios in the mesophilic control digester and raw feed were the same during Level 3. It was noted that the p values of these exceptions were small ($0.10<p<0.09$), likely as a result of the high variability of the feed ARG ratios. Statistical analysis using un-normalized ARGs (Figure 3, "#" symbol)

shows similar results as ARG ratios where *tet*(W) gene copy numbers in Levels 1, 2, and 3 and *tet*(O) gene copy numbers in Levels 1 and 2 were the same or higher in the mesophilic digester compared to raw sludge.

Effect of raw sludge ARGs on digester ARGs. ARG ratios and un-normalized levels in refrigerated (4 °C) raw sludge were observed to increase during the approximate four to six-week storage period. *sul*I and *int*I1 ARGs in the raw sludge increased as much as seven and four-fold, respectively, while the 16S bacteria rRNA less than doubled. *Sul*II, *tet*(O), and *tet*(W) genes did not increase. The Level 2 study period coincided with the use of "middle-age" to "old" sludge resulting in increasing *sul*I and *int*I1 ARG ratios in raw sludge feed with time and high variation. Transition from "old" to "new" feed sludge during the Level 3 sampling period similarly resulted in high variation in ARGs.

Increasing levels of ARGs in the raw sludge during extended refrigerated storage of the feed presented the opportunity to examine digester performance with increasing ARG levels in the feed. During two separate study periods, ARG ratio and copy numbers remained constant in both mesophilic and all thermophilic test and control digesters regardless of the feed ARG content (Figure 4, only Level 2 data for control digesters shown). It is not known whether it is the extended storage time , low temperature, lack of mixing, or a combination thereof that induces the increase in ARGs.

Discussion

Effect of Ag NPs on digester performance. Recent studies of Ag NPs in sludge have confirmed that Ag NPs readily bind with sulfide (Kaegi et al., 2011) and forms individual and small aggregates of silver sulfide nanoparticles (Kim et al., 2010). Based on these studies, it is likely that amended ionic silver and Ag NPs were rapidly and completely converted to sulfidized Ag.

While the fate of Ag NPs is now widely accepted, the mechanism of toxicity of Ag NPs is still under investigation in some cases with conflicting results. It is difficult to ascertain whether the antimicrobial properties of Ag NPs are caused by leaching of the silver ion, nanoscale interactions with bacteria based on size and morphology, or a combination thereof (Sotiriou et al. 2010; Morones et al., 2005, Pal et al., 2007). Reinsch et al. (2012) report that *Escherichia coli*

inhibition is reduced with increased sulfidation of Ag NPs. This study also highlights that Ag NPs can be sulfidized to different extents, i.e., fully or partially enveloped/passivated, and that variable levels of sulfidation impart differences in toxicity seemingly based on the remaining level of exposed Ag. For this reason, simply stating "sulfidized" or "non-sulfidized" may not be an adequate descriptor to describe the level of toxicity.

In this study, both Ag NPs and silver dosed up to a concentration of 1 mg-Ag/L did not have an observable impact on digester performance. It is possible that toxic effects were not observed because of the low concentrations of Ag NPs used in the study or the likely sulfidation of Ag NPs to form silver sulfide. The "no observed effect" of ionic silver on digester performance is not surprising. The toxicity of metallic or bulk silver is attributed to the aqueous concentration of the free ion, Ag^{+}. Consequently, processes that reduce the free ion concentration by binding with other elements or preventing the binding of the free ion to cells will reduce toxicity (Ratte, 1999). Lytle (1984) and Bard et al. (1976) found that silver was converted predominately to silver sulfide and accumulated in the sludge biomass during wastewater treatment. Indeed, the solubility product (Ksp) of silver sulfide is approximately 10^{-41}. Further, Bard et al. (1976) investigated the effect of various forms of silver on oxygen uptake rates by activated sludge biomass. The study found that 100 mg-Ag/L as silver sulfide did not inhibit respiration, while 10 mg-Ag/L as free ion derived from silver nitrate and silver chloride led to an 84% and 43% inhibition, respectively. This suggests that bound silver is not biologically active, while free silver ions and to a lesser extent aqueous complexes inhibit microbial activity.

Effect of SMX on digester performance. Published literature on the effect of antibiotics on anaerobic digestion and *Archaea* is limited (Hilbert et al., 1986; Sanz et al., 1996; Dridi et al., 2001). Therefore, only speculations can be made about the mechanistic effects of SMX on digester operations. The antibiotic action of SMX in bacteria is through competition with the precursor para-aminobenzoic acid (PABA) for the catalyst site on the enzyme dihydropteroate synthetase that converts PABA to a precursor of folic acid. DiMarco et al. (1990) reports that PABA is also a precursor in the biosynthesis of tetrahydromethanopterin (H4MPT), a cofactor in methanogensis. H4MPT (among other enzymes) catalyze the conversion of carbon dioxide to methane using hydrogen as electron donor in hydrogenotrophic methanogens (Rouviere and Wolf, 1988). Lessner (2009) states that H4MPT is a structural analog to the enzyme

tetrahydrosarcinapterin (H4SPT), which is an enzyme involved in the shuttling of carbon from acetate's carboxyl group to methane by acetoclastic methanogens. Furthermore, Dimitru et al. (2003) found a competitive relationship between PABA and various PABA-like inhibitors for the active site on an enzyme (namely 4-(β-d-ribofuranosyl)aminobenzene-5′-phosphate (RFA-P) synthase) that catalyzes the first step in the biosynthesis of H4MPT. Although not reported in any other literature, it is hypothesized that inhibition of H4SPT and H4MPT biosynthesis, in this case as a result of competition of SMX and PABA, results in reduced methanogenic activity of acetoclastic and hydrogenotrophic methanogens. This could explain the accumulation of acetic acid, other VFAs, and hydrogen observed in this study. However, Fountoulakis et al. (2004) reported that SMX up to 400 mg/L had no inhibitory effect on acetoclastic methanogens. The increase in gas volume could be the result of carbon dioxide stripping at the lower pH; however, there was no measured increase in the carbon dioxide proportion. The reason behind the increase in gas volume in the present study with no increase in carbon dioxide proportion is unclear.

Effect of Ag NPs and SMX on ARGs. The lack of a consistent trend of ARG copy numbers with ionic silver dosing concentration is not surprising because other studies predict removal of ionic silver from solution by sulfide binding and subsequent loss of toxicity (Ratte, 1990; Bard et al., 1976). The antimicrobial property of Ag NPs may also result from size-dependent nanoscale interactions with bacteria. However, Ag NPs at the environmentally relevant concentrations tested in this study (0.01 to 1.0 mg-Ag/L) also did not elicit an ARG response.

The lack of response of *sul*I and *sul*II ARGs to SMX is surprising, particularly at the dose above the MIC (Level 3 = 50 mg-SMX/L, ~5 g-SMX/kg TS). It seems logical that a high nutrient, microbially dense environment in the presence of selection pressures may result in an increase in ARGs. Indeed, studies have reported increased ARG transcription in response to even sub-MIC antibiotic levels (Davies et al., 2006). Studies investigating the fate of ARGs in dairy lagoons have also reported a positive correlation of ARGs with increased antibiotic concentrations (Pei et al., 2007; McKinney et al., 2010). Pei et al. (2007) conducted studies at 4°C and 20°C with SMX concentration of 20 mg/L, while McKinney et al. (2010) analyzed environmental samples collected at ambient temperatures over the four seasons (no specific temperatures reported) with SMX concentrations up to 840 µg/kg. In addition, it is widely accepted that the mesophilic

gastrointestinal tract of humans and farm animals also gives rise to ARBs under antibiotic selection pressures via mutation or horizontal transfer (Levy and Marshall, 2004).

Correlation of intI1 with ARGs. Class 1 integrons are mobile genetic elements that often contain multiple ARGs. Selection for one ARG may result in the selection of all ARGs housed on the integron. As such, *int*I1 could be an important indicator of ARG propagation. *Int*I1 in digested sludge had significant correlation ($p<0.05$) with all genes analyzed, although the strength of the correlation varied between genes as strong (*sul*I, $R^2 = 0.74$), moderate (*sul*II, $R^2 = 0.66$ and *tet*(O), $R^2 = 0.55$), and weak (*tet*(W), $R^2 = 0.04$). Strong correlation of *sul*I and *int*I1 is not surprising because *sul*I is typically associated with Class 1 integrons (Mazel, 2006).

Effect of digestion temperature on ARGs. In comparing the thermophilic and mesophilic control digesters, the ARG response is gene specific. The results of this study suggest that thermophilic digestion and mesophilic digestion have similar removals of *sul*I, *sul*II, and *int*I1 genes ranging from one to two log reductions. Thermophilic digesters consistently reduced *tet*(O) and *tet*(W) genes as well, while at times in the mesophilic digester these genes increased relative to the feed. Ma et al. (2011) and Diehl and Lapara (2010) have also reported gene-specific responses to digestion temperature with respect to *tet* genes. Diehl and Lapara (2010) also reported temperature dependence with the *int*I1 gene; however, this study and Ma et al. (2011) found consistent reduction of the *int*I1 gene in either mesophilic or thermophilic digestion.

Ahring et al. (2001) reported that at 55°C, bacterial rRNA contributed 74 to 79% and archaeal contributed 18 to 23% of the universal probe, whereas at 65°C, the archaeal contribution increased to 34 to 36% and the bacterial contribution decreased to 57 to 62%. In general, the proportion of the microbial community occupied by *Archaea* increases with increasing temperature while the proportion occupied by *Bacteria* decreases (Ahring et al., 2001). *Archaea* are resistant to most antibiotics because their physiology and structure differ from bacteria such that the antibiotic targets are not recognized. As such, an increase in *Archaea* population relative to *Bacteria* would reduce the total number of possible hosts and ARG embodied therein. However, a 16S bacterial rRNA primer was used in this study, so a reduction in the ARG ratio reflects the reduction of ARG copy numbers relative to bacteria. This suggests that the number of ARGs per bacterial cell were reduced overall, not just reduced due to the reduction of bacterial

population relative to the archaeal population. Although the shift in microbe composition between *Bacteria* and *Archaea* may account for some loss of total ARGs, this loss should have been accounted for when normalizing ARG copy numbers to 16S bacterial rRNA gene copy numbers.

Effect of raw sludge ARG content on digester ARG content. Un-normalized ARGs and ARG ratios remained constant in both mesophilic and thermophilic control digesters during addition of raw sludge that varied in the levels of *intI1* (Figure 4). However, Figures 2 and 3 show a similar pattern of ARG content in the raw sludge feed and digesters. For example, *sul*I and *intI1* levels in the raw sludge increased from Level 1 through Level 3, which covers approximately six months. Similarly, *sul*I and *intI1* levels in the digested sludge from all digesters also increased from Level 1 through Level 3 time periods. More investigation is required to determine the impact of raw sludge ARG content on digester ARG content.

***ARG Attenuation Mechanisms*.** In digestion, the fate of ARGs depends on the cumulative effect of the following: proliferation/attenuation of ARGs with replicating/dying raw sludge bacteria or digester bacteria, differential replication of ARG-containing raw sludge or digester bacteria (enrichment), horizontal transfer of ARGs from raw sludge bacteria to digester bacteria, and mutation. Mutation may be discounted in this study because it is unlikely that mutations would give rise to the specific genes recognized by the QPCR assays.

It appears that the higher temperatures and/or endogenous conditions used in mesophilic and thermophilic anaerobic digestion may create an environment that is not conducive to horizontal transfer or may support a microbial community that is not compatible with incoming raw sludge bacteria for horizontal transfer of the ARGs. Indeed, diversity among bacteria is reduced in thermophilic digestion relative to mesophilic digestion (LaPara et al., 2000; Leven et al., 2007; Wilson et al., 2008). Thus, as pointed out by Ma et al. (2011), restriction of the host range may play a part in attenuation of ARGs in thermophilic digestion. In addition, it also seems plausible that the differences in the microbial community are the reason behind varied individual gene response to mesophilic and thermophilic conditions.

Conclusions

The findings of this study include:

- **Effect of Ag NPs and SMX on digester performance**: Neither direct application of Ag NPs nor ionic silver affected digester performance as assessed by pH, alkalinity, VFA, and VSR. Low concentrations of antibiotic SMX (<5 mg/L) did not affect digester performance; however, the highest dose (50 mg/L) resulted in digester souring, where pH and alkalinity decreased as a result of the accumulation of VFAs. It is possible that SMX inhibits the synthesis of methanopterin, a key enzyme in hydrogenotrophic methanogenesis.

- **Effect of Ag NPs and SMX on ARGs**: ARG ratios and un-normalized copy numbers did not increase relative to the thermophilic control in response to the amendments added to test thermophilic digesters. In general, ARG ratios and un-normalized copy numbers were the same (*sul*I, *sul*II, and *int*I1) or higher (*tet* ARGs) in mesophilic digestion (37 °C, 20-day SRT) than thermophilic digestion (53 °C, 12-day SRT) when using the same feed.

- **Effect of digestion temperature on ARGs**: Test and control thermophilic digesters consistently reduced, but did not eliminate, levels of all genes that were analyzed (*sul*I, *sul*II, *int*I1, *tet*(O), and *tet*(W)). The mesophilic digester also reduced levels of *sul*I, *sul*II, and *int*I1 genes, but levels of *tet*(O) and *tet*(W) were the same or higher than in raw sludge.

- **Effect of raw sludge ARG content on digester ARG content**: ARG ratios in digested sludge remained constant under increasing concentration of feed ARGs. Quantities of the five ARGs in the raw sludge ranged from 10^4 to 10^8 copies per microliter of sludge, where quantities of *sul*II, *tet*(O), *tet*(W) were consistently one order of magnitude lower than quantities of *intI1* and *sul*I. Quantities in digested sludge ranged from 10^3 to 10^6 as a result of one log reduction of ARGs (two log reduction for *int*I1) during anaerobic digestion.

In summary, this study shows that mesophilic and thermophilic anaerobic digestion reduces, but does not eliminate, ARG loading to the environment. Thermophilic digestion reduces a wide variety of ARGs, whereas tetracycline genes are not reduced and sometime increase in mesophilic digestion. The fact that ARG ratios in digested sludge (reactor effluent) remained

constant in spite of the application of selection pressures and variable feed ARG concentrations suggest that the digester operating conditions themselves are the major factor that governs the composition of the bacterial community and thus the prevalence of ARGs. Future studies are suggested that investigate the impact of stress conditions on ARG horizontal transfer and persistence and the fate of ARGs post-digestion.

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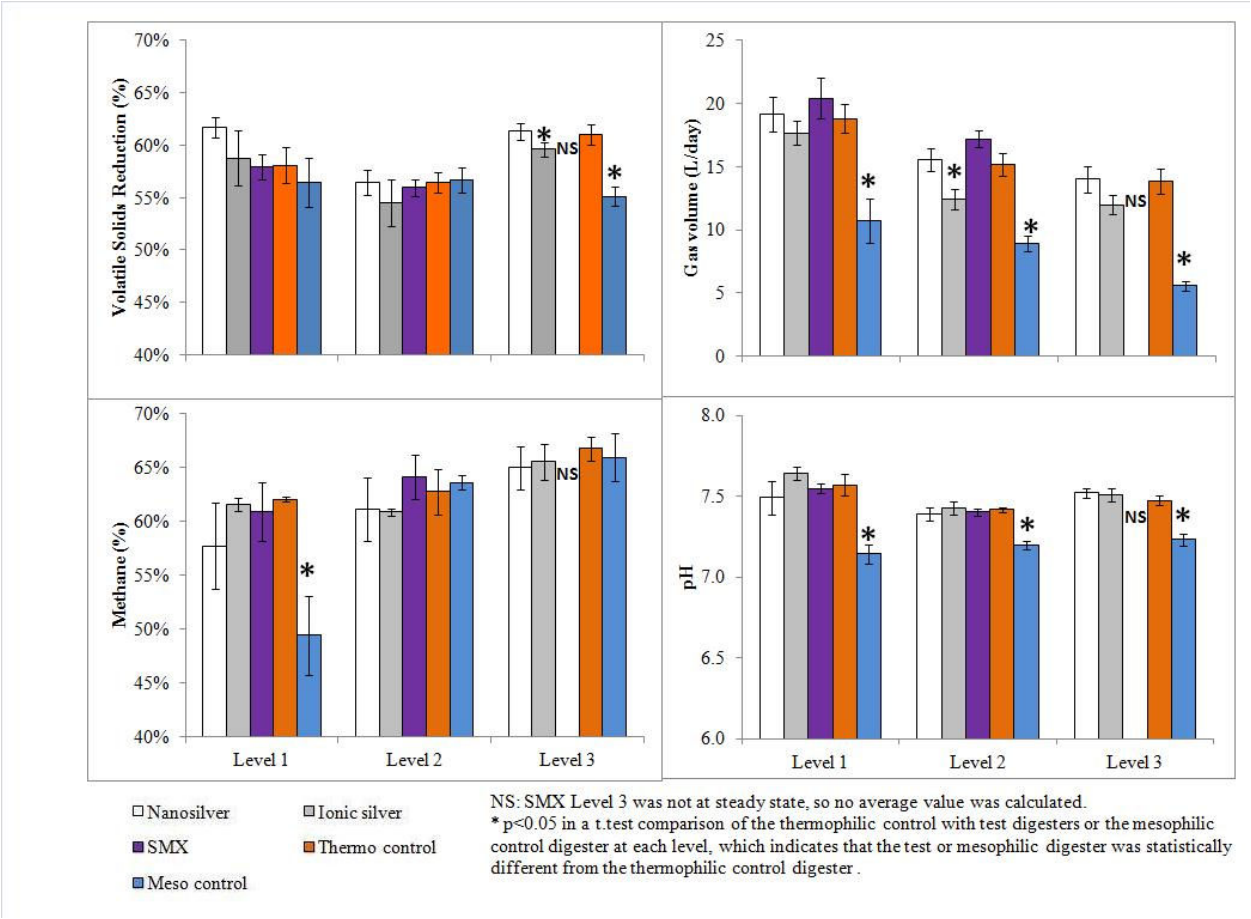

Figure 4-1. Comparison of volatile solids reduction (%, VSR), pH, gas volume, and methane composition during all dosing levels. Error bars represent standard error of three to six samples collected at steady state every three days over 1.5 to 2 SRTs for Levels 1, 2 and 3.

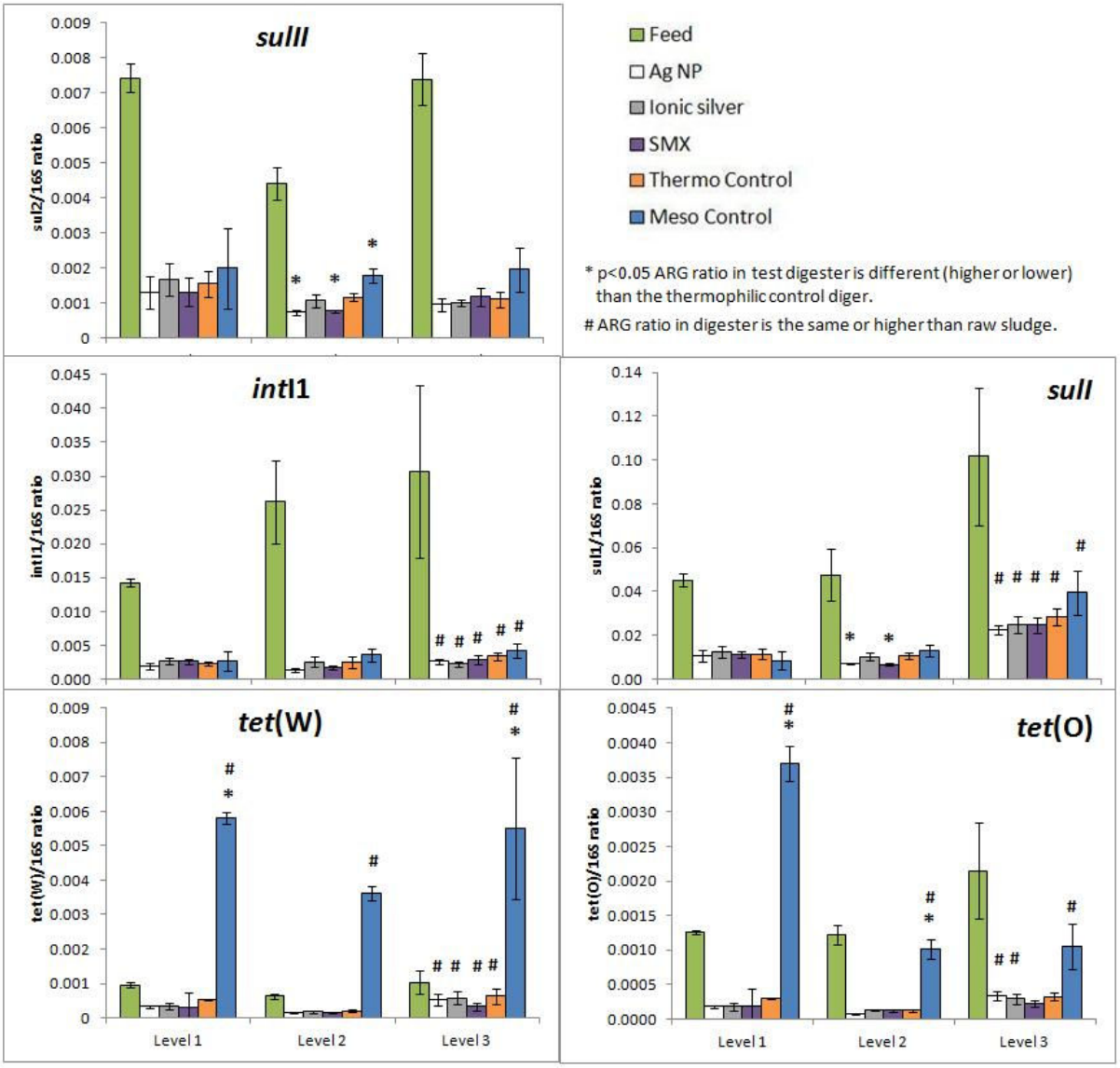

Figure 4-2. ARG and *int*I1 ratios in the raw feed sludge (Feed), test digesters (Ag NP, Ionic silver, and SMX), thermophilic control digester (Thermo Control), and mesophilic control digester (Meso Control). Error bars represent standard error of four to six samples collected at steady state every three days over 1.5 to 2 SRTs for Levels 1, 2 and 3.

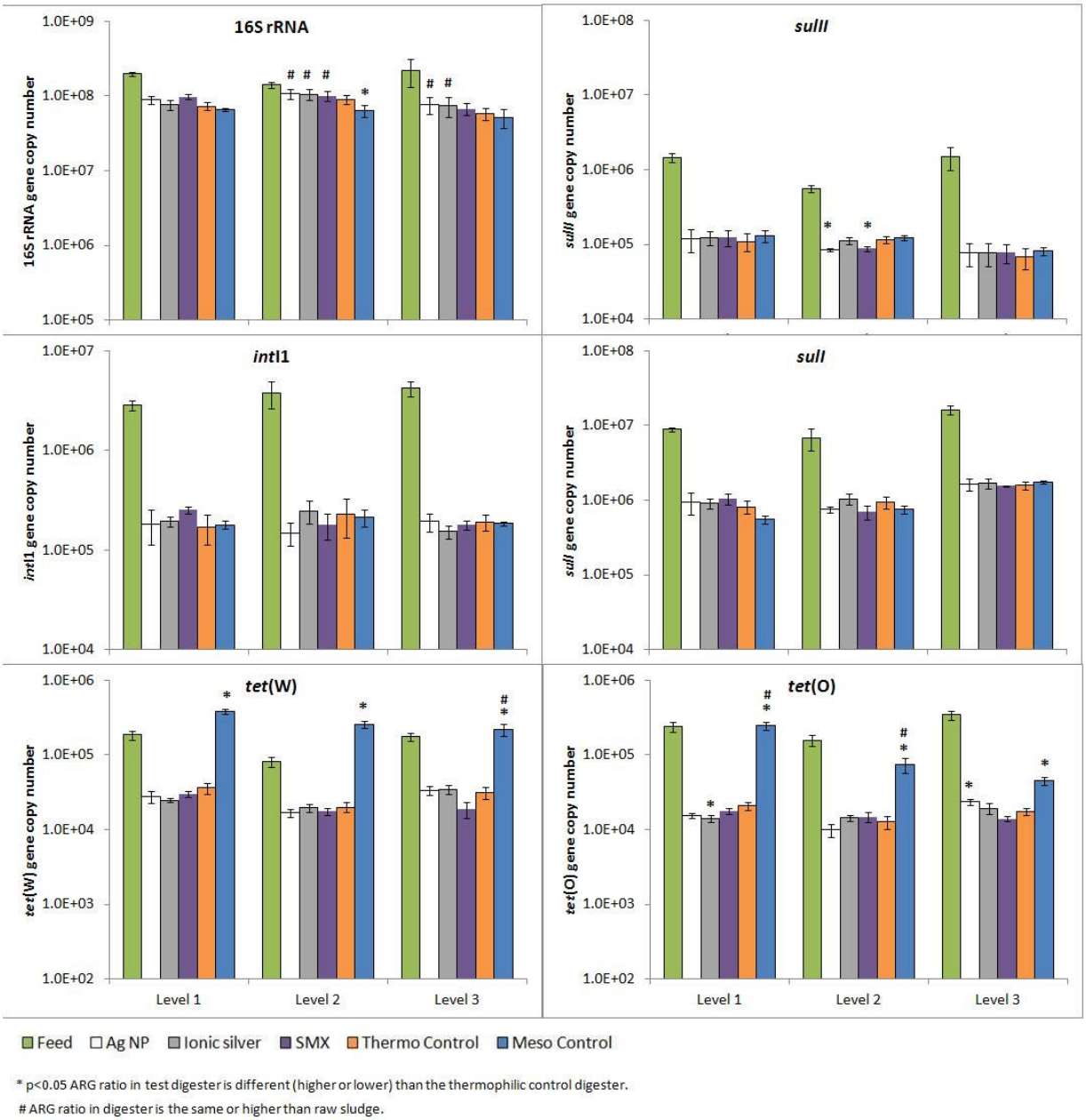

Figure 4-3. Quantities of ARGs and *int*I1and 16S rRNA gene copy numbers (number per µl sludge) in the raw feed sludge (Feed), test digesters (Ag NP, Ionic silver, and SMX), thermophilic control digester (Thermo Control), and mesophilic control digester (Meso Control). Error bars represent standard error of four to six samples collected at steady state every three days over 1.5 to 2 SRTs for Levels 1, 2 and 3.

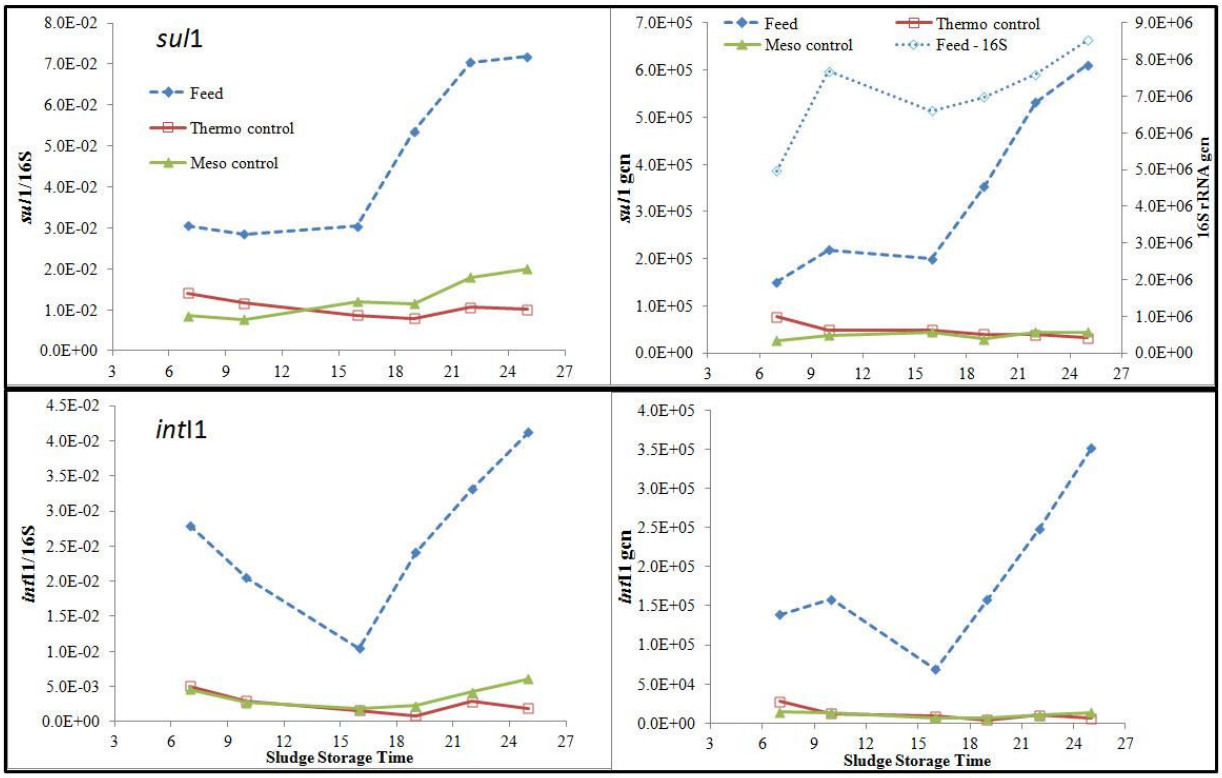

Figure 4-4. *sul1* and *int*I1 gene copy numbers and ARG ratios during the Level 2 steady state sampling period. Each data point represents a single DNA extraction from one sample collected from the respective digester or feed on the given day. 16S rRNA, *int*I1, and *sul1* gene copy numbers were analyzed via QPCR in triplicate on aliquots from the same extracted DNA.

CHAPTER 5

ARG increase in response to cold temperature exposure: Implications for winter storage of sludge and biosolids

Jennifer H. Miller, John T. Novak, William R. Knocke, Amy Pruden

Significance and Impact of the Study

Wastewater treatment plants have been identified as hotspots for the proliferation and dissemination of antibiotic resistance genes (ARGs) and antibiotic resistant bacteria (ARBs) to the environment through discharge of treated effluent to water bodies as well as application of biosolids to land. Identifying critical control points within the treatment process may aid in the development of solutions for the reduction of ARGs and ARBs and curbing the spread of antibiotic resistance. This study found increases in ARGs during biosolids storage and identifies changes in operational protocols that could help reduce ARG loading to the environment when biosolids are land-applied.

Introduction

It is common practice to store treated sludge in tanks, basins, or lagoons until a time suitable for land application. Rain, snow, frozen ground, spring thaw, and cropping practices typically delay opportunities for land application for weeks or months, particularly in northern climates. In one extreme example, Martel (2001) designed a dewatering bed for sludge storage at McMurdo station, Antarctica during the 6-month winter between March and September.

Miller et al. (2013) observed increased concentrations of an antibiotic resistance gene, *sul1* (encoding sulfonamide resistance) and the *int*I1 gene (encoding the integrase gene of Class 1 integrons), in a mixture of raw primary and secondary sludge that was stored at 4 °C prior to feeding lab-scale anaerobic digesters. *sul*1 is often co-located on Class 1 integrons (Paulsen et al., 1993). No effect was observed on tetracycline resistance genes, *tet*(O) and *tet*(W), which are not associated with integrons (Roberts, 2005; Recchia and Hall, 1995), but have been found on chromosomes, plasmids, and transposons (Roberts, 2005). Full-scale sludge storage at a wastewater treatment plant, particularly during the winter, presents similarities with the storage

conditions observed in the lab - namely, low temperatures, lack of mixing, lengthy storage times, and anaerobic biological activity. As such, an investigation into the fate of ARGs during winter storage of sludge or biosolids was initiated with reduced temperature hypothesized to have a significant effect on ARG proliferation.

Previous studies suggest potential mechanisms for the above hypothesis. For example, the bacterial response to cold shock is a regulatory cascade of gene transcription collectively termed the general stress response, or rpoS response (White, 2007). The general stress response is characterized by increased production of the rpoS sigma factor, which enables binding of RNA polymerase to the promoters of genes that may be required to respond to the detected stress. Various stresses are known to induce the rpoS response, such as starvation, out of range pH, osmotic shock (high salt), heat shock, high hydrostatic pressure (Vanlint et al., 2003; Wemekamp, 2002), and cold shock. Pertinent to the spread of antibiotic resistance, multiple studies have shown that in an effort to increase genetic diversity in times of stress, the rpoS response increases the competence and transformation capability of bacterial cells for the uptake of DNA (Claverys et al., 2006; Capy et al., 2000; Ilves et al., 2001). Numerous studies have shown that low temperatures specifically result in increased competence (Turner et al. 1990; Carr, et al., 2010; Kretschmer and Cohen, 1979).

The SOS response, coined after the distress signal in Morse code, is often described as the cellular response to DNA damage, such as from UV light, chemicals, oxidative stress, or antibiotics (Miller et al. 2004; Maiques et al. 2006). The list of potential triggers for the SOS response is expanding and overlaps with triggers for the rpoS response, with new research including environmental stresses (Aertsen, 2006), such as starvation (Bernier et al.., 2013), heat shock (Layton et al., 2005) and high hydrostatic pressure (Aertsen et al., 2004).

The SOS response also induces increased rates of genetic recombination and horizontal gene transfer as reported with a mobile genetic element responsible for the multi-drug resistance of *Vibrio cholera* (Beaber et at., 2004) and Class 1 integrons (Guerin et al., 2009; Cambray et al., 2011) . Class 1 integrons are one of five classes of mobile integrons that have had a profound impact on the spread of antibiotic resistance. Class 1 integrons group multiple ARGs under a common promoter and are associated with mobile genetic elements, such as insertion sequences,

conjugative plasmids, or transposons (Mazel, 2006). Numerous studies have found Class 1 integrons at wastewater treatment plants (Ghosh et al., 2009; Guardabassi et al., 2002; Kim et al., 2007; Zhang et al., 2009). While Beaber et al. (2004) found a direct link between the SOS response and transcription of genes responsible for the excision and transfer of the mobile genetic element, the findings of Guerin et al. (2009) appear limited to enhanced genetic recombination within the integron and not necessarily enhanced transfer rates. However, the relationship between environmental triggers of bacteria stress responses, gene recombination, and horizontal gene transfer has much to be explored (Baharoglu et al., 2012).

In summary, cold temperatures may impact ARG horizontal gene transfer through mechanisms such as enhanced cell competence and transformation associated with the general stress response mechanisms (rpoS) and enhanced genetic recombination and transfer associated with the SOS response. The potential link between ARG levels in biosolids and stresses, such as cold shock, has not previously been studied to the knowledge of the authors.

The purpose of this study was to investigate the response of the integrase enzyme (*int*I1) of Class 1 integron and *sul*1, an ARG commonly located on Class 1 integrons that encodes resistance to sulfonamides, to temperatures that may initiate enhanced horizontal gene transfer as a stress response. In this study, Class B mesophilic digested sludge was stored at 4 °C, 10 °C, and 20 °C for a period of 4 months. Each month, TS, VS, pH, alkalinity, *sul*1, and *int*I1 were measured to evaluate the impact of storage temperature on ARG fate.

Results and Discussion

Dewatered mesophilic digested sludge (biosolids) was used to establish replicate storage microcosms that were subsequently stored at three temperatures (4 °C, 10 °C, 20 °C) for a period up to four months. At monthly intervals, three replicates from each temperature set were sacrificed and analyzed for TS/VS, pH, alkalinity, and quantitative polymerase chain reaction (QPCR) analysis of *int*I1 and *sul*1. These ARGs were selected because *int*I1 encodes the integrase enzyme for Class 1 integrons, a highly mobile genetic element that potentially groups multiple ARGs under a common promoter (Mazel, 2006) including *sul*1, which is frequently located in the conserved region of Class 1 integrons (Paulsen et al., 1993). ARGs were normalized to 16S rRNA, TS, and VS. Normalization to 16S rRNA typically accounts for

variability in DNA extraction efficiencies and biomass between samples. Normalized ARG ratios can be thought of as roughly representing the percentage of bacteria containing the ARG in a given sample, but normalization can mask increases or decreases in ARG copy numbers as a result of biomass growth or death. In addition, ARGs were normalized to TS and VS because the water contents of biosolid microcosms were variable at collection time because of continued solids reduction and uneven addition of sterilized nanopure water to prevent drying of samples during storage. Paired t tests were used to compare log ARGs, ARG 16SrRNA ratios, and log ARG TS/VS ratios in the biosolids at the start of the study (Day 0) and at each temperature/storage time triplicate. Probability (P) values for all t tests and unnormalized intI1, sul1, 16S rRNA, TS, and VS are provided in Supporting Information.

In biosolid samples stored at 4 °C, a 3-log increase in unnormalized *sul*1 concentrations (Figure S2) spanning the time between Day 0 and 2 months resulted in a statistically ($P<0.05$) significant increase in normalized ratios of *sul*1 to TS (Figure 1a), VS (Figure 1a), and 16S rRNA (Figure 1b) at 1 month and 2 months. The trend in normalized *int*I1 ratios mirrored normalized *sul*1 ratios, but only the normalized ratio of *int*I1 to 16S rRNA at 2 months was significantly higher than the Day 0 ratio. Normalized gene ratios for both *sul*1 and *int*I1 returned to initial (Day 0) levels by four months.

Average pH for each temperature/storage time triplicate are shown on Figure 1a. The pH increased in all triplicates (pH 8.3, 8.2, and 8.2 for 1 month, 2 month, and 4 month triplicates, respectively) relative to Day 0 (pH 7.3) suggesting that the low temperature inhibited fermentation reactions that typically decrease pH during long term sludge storage.

In biosolid samples stored at 10 °C, ratios of *int*I1 and *sul*1 normalized to 16S (Figures 2b and 2b) increased with time, with the largest increase from initial *sul*1 ratios occurring at 2 months ($P<0.05$) and *int*I1 ratios at 1 month ($P=0.18$). The increase in VS normalized *int*I1 ratio at 1 month was significant ($P<0.05$, Figure 2a). pH (Figure 2a) increased between Day 0 (pH 7.3) and 2 months (pH 8.1), then decreased substantially by 4 months (pH 6.9). It appears that the pH trend is a "slower" version of the pH trend observed at 20 °C and is likely a result of the cumulative effects of acid-producing fermentation reactions throughout the study.

The biosolid aliquots stored at 20 °C showed the least amount of increase in ARG ratios, although a significant increase ($P<0.05$) was observed at the 1-month time point for TS/VS normalized *sul*1 (Figure 3a) and at the 1-month and 2-month time points for 16S rRNA normalized *sul*1 (Figure 3b). Levels returned to background by 4 months. No comparisons between *int*I1 ratios in stored samples and the initial biosolid sample were significantly different ($P>0.05$). pH values (Figure 3a) increased between Day 0 (pH 7.3) and 1 month (pH 7.9), then decreased substantially at 2 months (pH 6.3) and 4 months (pH 5.9).

These figures illustrate that the highest increases in ARG ratios occur at a storage temperature of 4 °C, with modest increases in ARG ratios during sludge storage at all temperatures. The highest increases occurred between 0 and 2 months for all storage temperatures. Storage at 20 °C had the smallest increase in ARGs over time, followed by 10 ºC. Unnormalized ARG increases were less than 0.5 log for biosolids stored at 10 °C and 20 °C, but increases were between 0.5 log and 3 log for biosolids stored at 4 °C.

Given that the 16S rRNA concentration remained constant across all months and all temperatures, it is likely that the ARG increase is due to horizontal gene transfer or preferential survival of resistant organisms, rather than overall bacteria regrowth. However, it is not possible in this study to directly distinguish the relative impact of horizontal gene transfer and the survival and growth of resistant organisms over nonresistant organisms. Further work would be necessary to differentiate the two persistence mechanisms.

Considering the global epidemic of antibiotic resistance, evaluating hotspots of ARG dissemination such as wastewater treatment plants with an eye toward ARG reductions is of grave importance for reduction of ARGs in the environment as a whole. ARGs present in biosolids destined for land application represent a direct route of ARG release to the environment. This study has shown that the short term (1 to 2 month) storage of biosolids at temperatures ranging from 4 °C to 20 °C results in a 0.5 to 3-log increase in ARGs and a corresponding increase in normalized ARG ratios to 16S rRNA, TS, and VS. This work would suggest that minimal storage times between 2 and 4 months may help maintain ARGs at post-digestion levels, particularly for biosolids stored at low temperatures; however, it is recognized that increasing mandatory storage times increases storage basin size and cost.

Materials and Methods

Experimental Setup. Dewatered (belt thickener) Class B mesophilic digested sludge (biosolids) was obtained from the Christiansburg, Virginia Wastewater Treatment Plant in August 2012. Total and volatile solids, pH, and alkalinity were measured at this time and a sample was collected and frozen at -80 ºC for later QPCR analysis. Biosolids (200ml) were apportioned into 500ml HDPE bottles and covered loosely with a cloth bag filled with granular activated carbon for odor control and to capture any gas release. Sets of fifteen bottles were placed in i) 4 °C walk-in refrigerator, ii) 10 ºC constant temperature refrigerator, or iii) fume hood located at room temperature (20 ºC). Sterilized nanopure water was added twice a month to replace water lost by evaporation. Bottles were not shaken or stirred and solids were allowed to settle. Every month, three bottles were removed from each storage temperature set and analyzed for total and volatile solids, pH, alkalinity, and samples were frozen at -80 °C for later QPCR analysis.

Water quality monitoring. Total and volatile solids (Method 2540-G), total alkalinity (Method 2320-G), and pH (Method 4500) were analyzed as specified in Standard Methods for the Examination of Water and Wastewater (APHA, 1995).

Quantification of ARGs. DNA was extracted from 250 µL of digested sludge from the digesters or raw feed sludge (250 mg centrifuged sludge for Baseline samples) using FastDNA Soil Kit (MP Biomedicals, Solon, Ohio) according to manufacturer's protocol. Extracted DNA was diluted 50x to minimize inhibitory effects as determined by QPCR analysis of multiple dilutions. Diluted DNA extracts were stored at -20°C until analysis by QPCR for bacterial 16S rRNA genes, *sul*1, *tet*(O), *tet*(W), and *int*I1. A 10 µL reaction mixture was comprised of 5.0 µL SsoFast Evagreen Supermix (Bio-Rad, Hercules, California), 0.8 µL of each 5 µM primer (Ma et al., 2011), 2.4 µl molecular biology grade water, and 1 µL of DNA template. All samples were quantified in triplicate. Standards prepared from serial dilutions of cloned genes ranged from 10^2 to 10^8 gene copies per µL. Standards and a reagent blank were included in each run.

Statistics. Microsoft Excel 2007 was used to calculate averages and standard error of all data. R 2.8.1 (http://cran.r-project.org/bin/windows/base/old/2.8.1) was used to perform t tests on log ARG gene copy numbers, log ARG normalized to TS and VS ratios, and ARG to 16S rRNA ratios between the initial biosolid triplicates (Day 0) and each storage temperature/time

experimental triplicates. A P-value of <0.05 was considered to indicate a significant difference between means. All statistical results can be found in Supporting Information (Table S1).

Acknowledgements

The author would like to acknowledge the contribution of Emily Lipscomb for performing the DNA extractions. This work was supported by a fellowship from DC Water, the National Science Foundation Chemical, Bioengineering, and Transport Systems CAREER award #0852942, and Virginia Tech Institute for Critical Technology and Applied Science seed funding and award TSTS 11–26. Jennifer Miller was supported by the Charles E. Via, Jr. Department of Civil and Environmental Engineering Via Scholarship and Virginia Tech Graduate School Cunningham Fellowship. The findings of this study do not necessarily reflect the views of the supporting entities.

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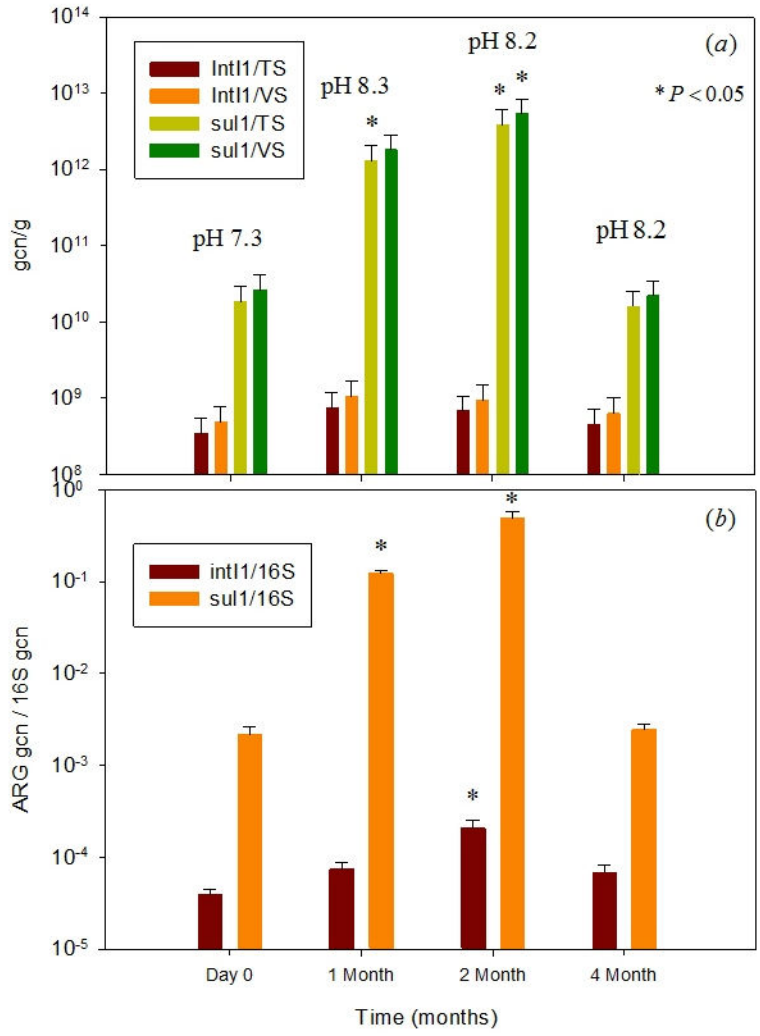
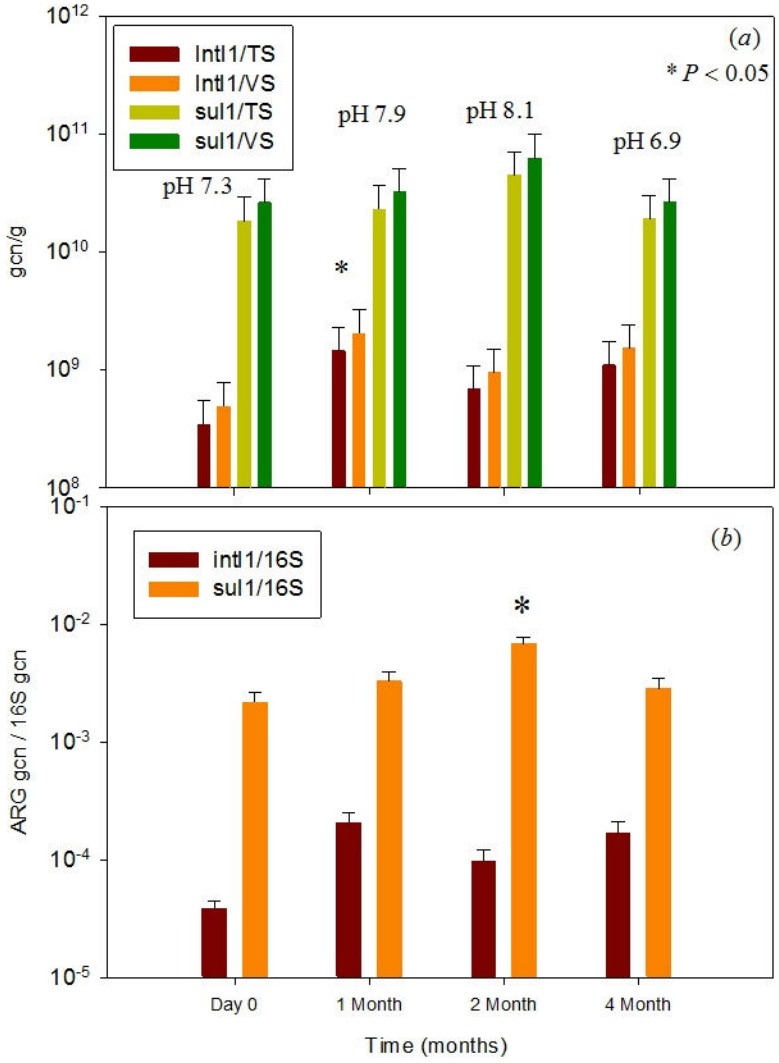
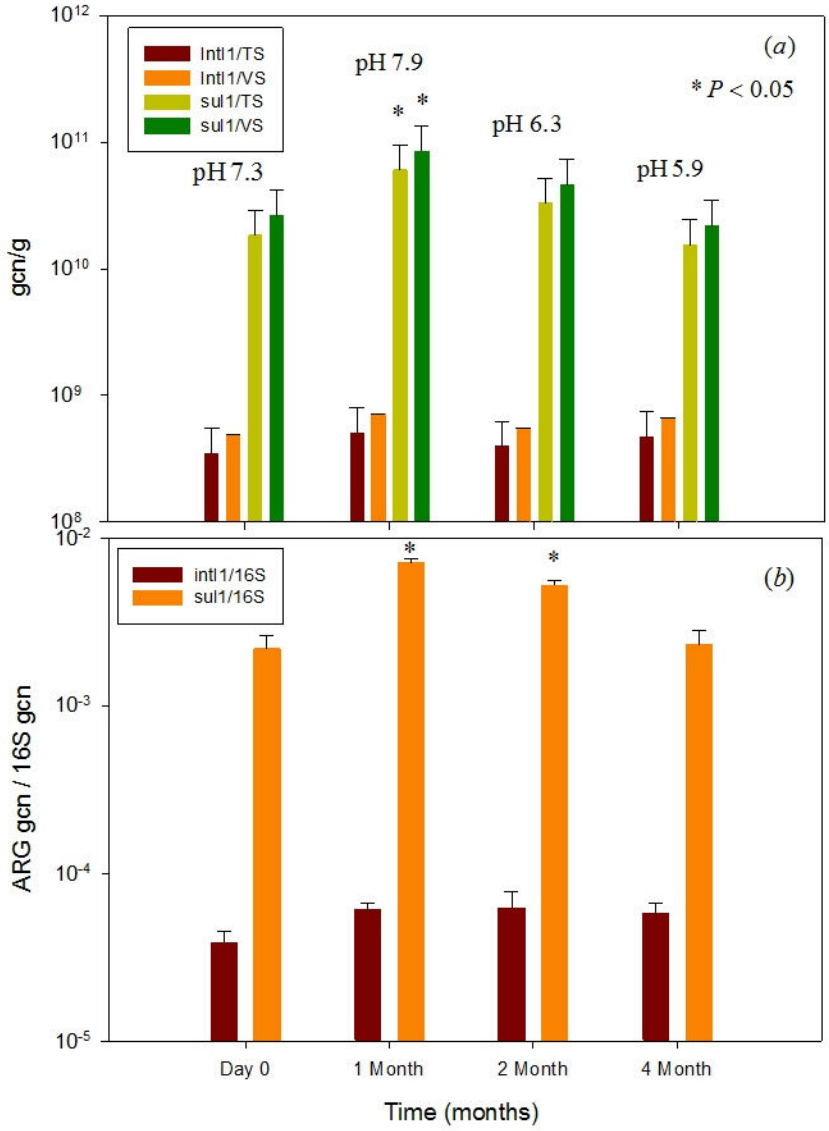
Figures



Figures 5-1a and 5-1b. For biosolids stored at 4 °C, ARG (*int*I1 and *sul*1) gene copy numbers normalized to TS (Figure 1a), VS (Figure 1a), and 16S rRNA (Figure 1b) expressed in units of gene copy numbers per kg solids (gcn/kg). Asterisks (*) denote P<0.05 in a statistical comparison Day 0 triplicates with a temperature/time triplicate. Average pH of the triplicates are given in parentheses above each data set.



Figures 5-2a and 5-2b. For biosolids stored at 10 °C, ARG (*int*I1 and *sul*1) gene copy numbers normalized to TS (Figure 2a), VS (Figure 2a),, and 16S rRNA (Figure 2b) expressed in units of gene copy numbers per kg solids (gcn/kg). Asterisks (*) denote P<0.05 in a statistical comparison Day 0 triplicates with a temperature/time triplicate. Average pH of the triplicates are given in parentheses above each data set.

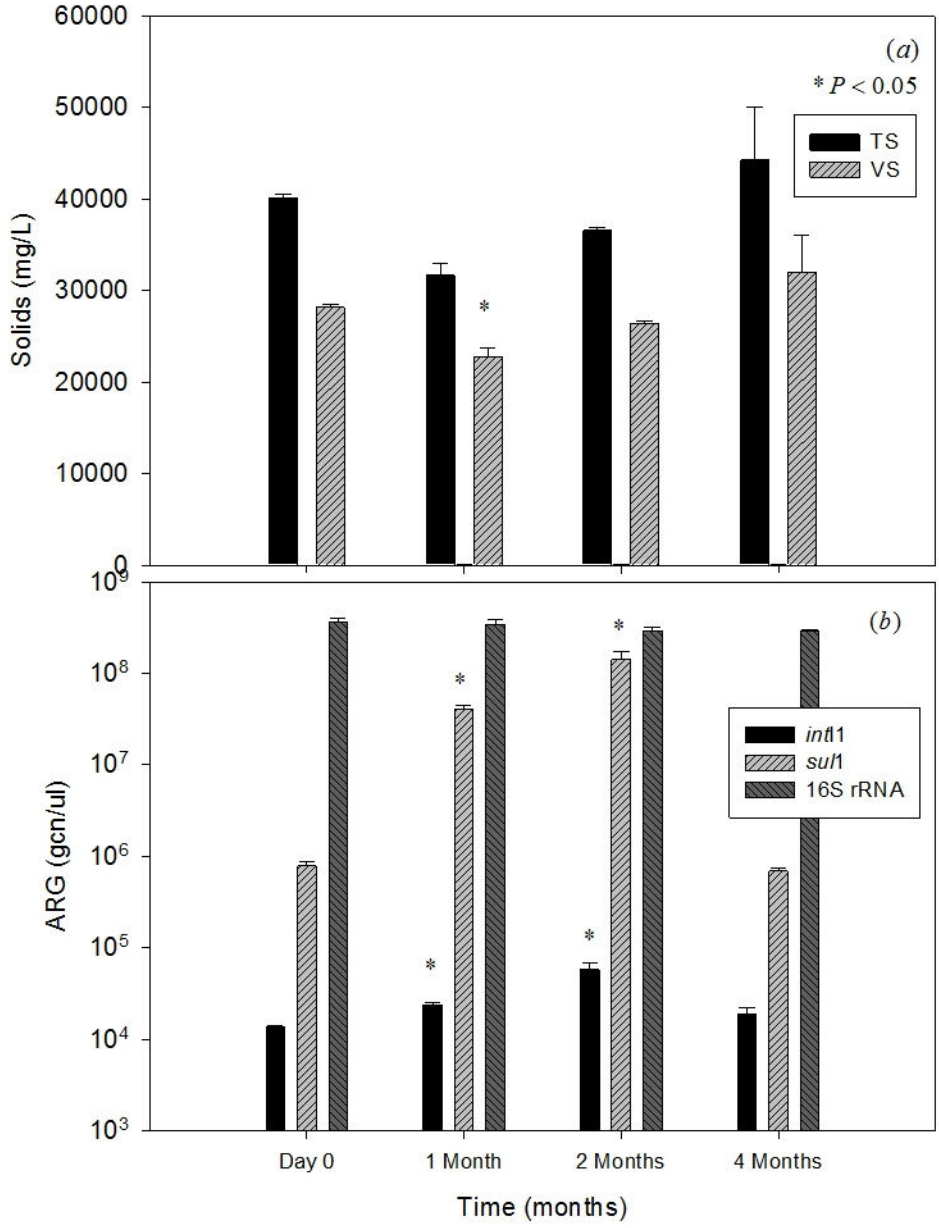


Figures 5-3a and 5-3b. For biosolids stored at 20 °C, ARG (*int*I1 and *sul*1) gene copy numbers normalized to TS (Figure 3a), VS (Figure 3a), and 16S rRNA (Figure 3b) expressed in units of gene copy numbers per kg solids (gcn/kg). Asterisks (*) denote P<0.05 in a statistical comparison Day 0 triplicates with a temperature/time triplicate. Average pH of the triplicates are given in parentheses above each data set.

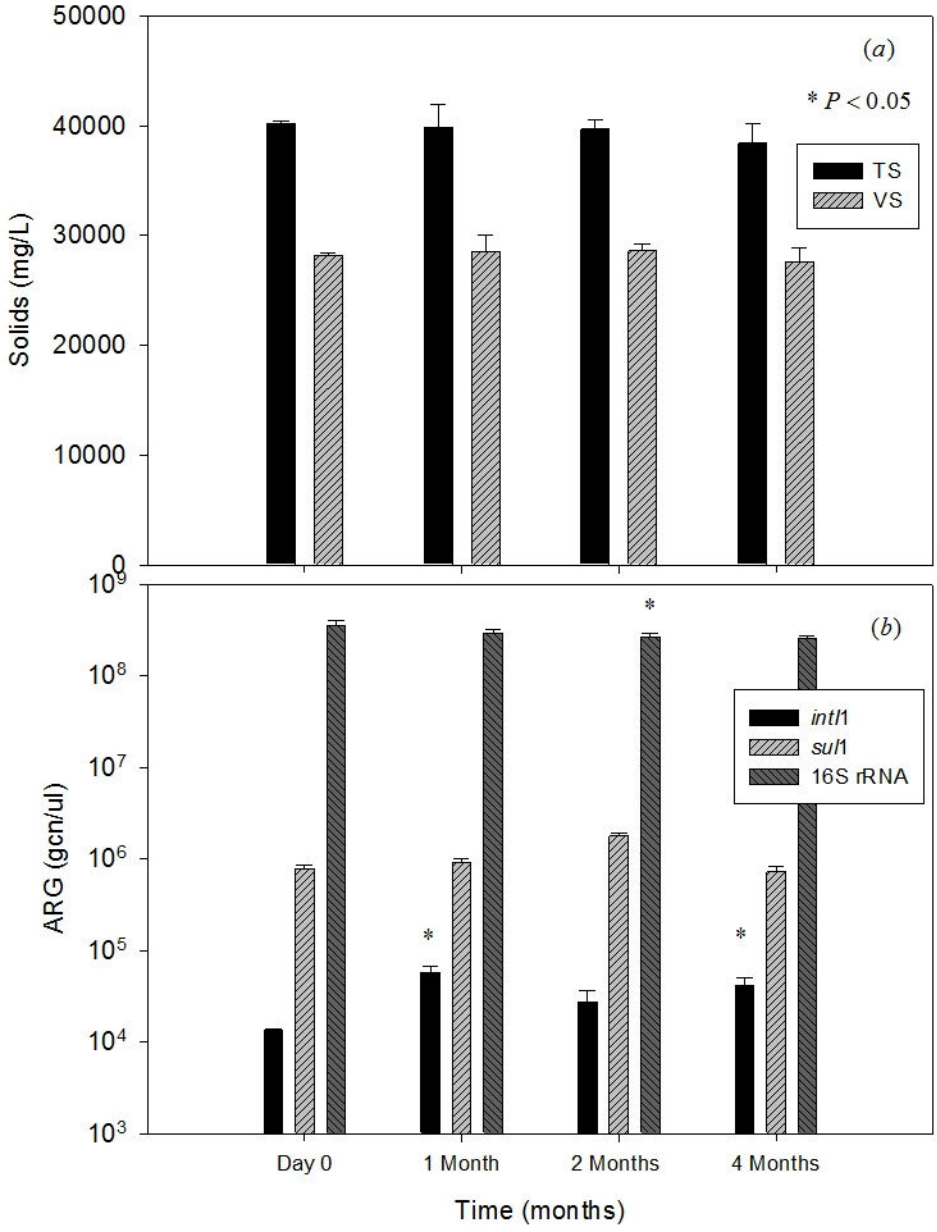
Supporting Information

Table S5-1. Probability (P) values t tests on log ARG gene copy numbers, log ARG to TS and VS ratios, and ARG to 16S rRNA ratios between the initial biosolid triplicates (Day 0) and storage temperature/time experimental triplicates. A P-value of ≤0.05 was considered to indicate a significant difference between means.

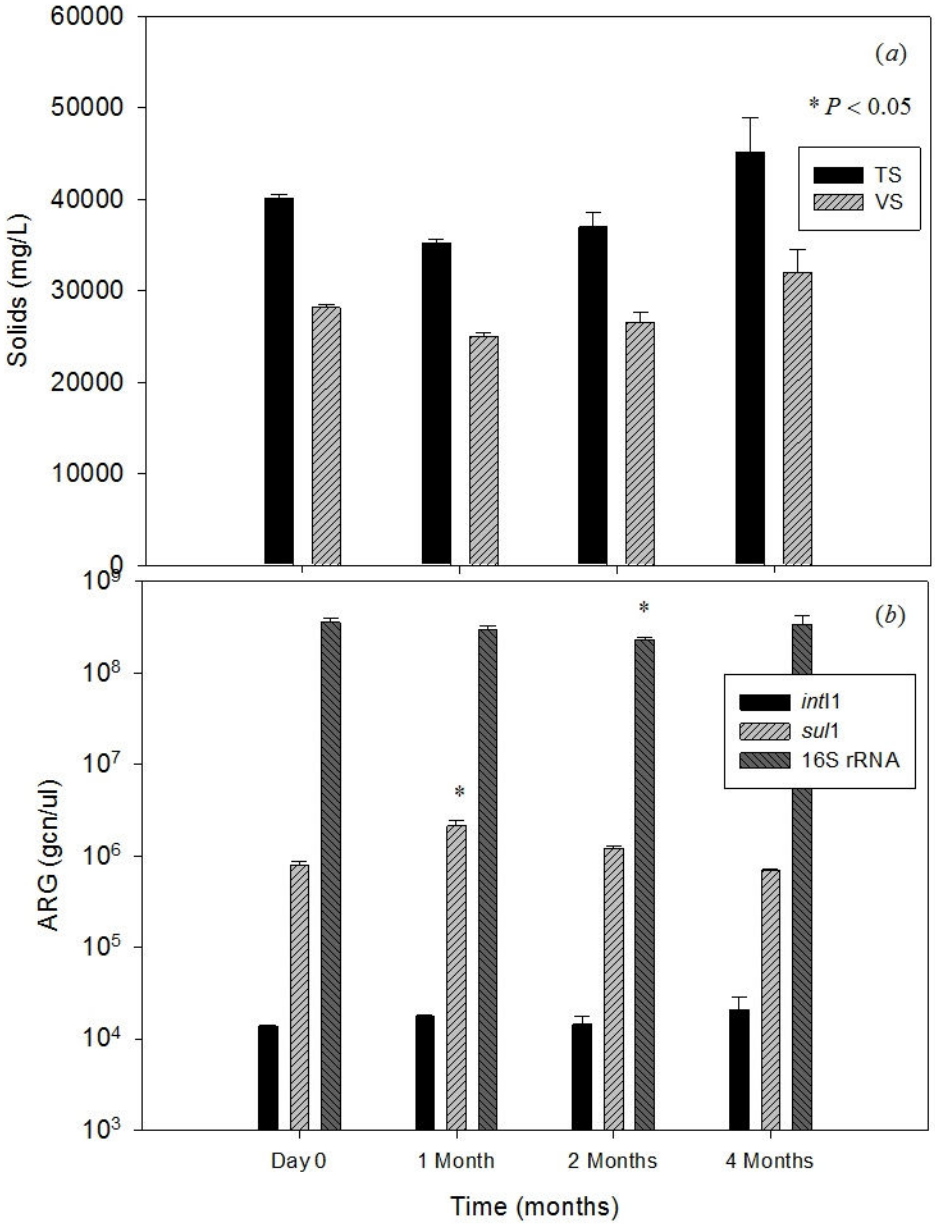
	log (VS)	log (TS)	log (*sul* 1)	log (*int* I1)	log (16S rRNA)	log (*int* I1/TS)	log (*int* I1/VS)	intI1/ 16S rRNA	log (*sul* 1/TS)	log (*sul* 1/VS)	*sul* 1/ 16S rRNA	pH
4 °C, t test pairing Day 0												
1 Month	0.042	0.111	0.0004	0.073	1	0.079	0.073	0.174	0.002	1	0.004	0.006
2 Month	0.126	1	0.003	0.020	0.423	0.058	0.073	0.035	0.050	0.051	0.033	0.003
4 Month	0.416	0.451	0.667	0.383	0.184	0.868	0.933	0.238	0.728	0.697	0.733	0.014
10 °C, t test pairing Day 0												
1 Month	0.742	1	0.423	0.009	0.184	0.037	0.038	0.075	0.530	0.564	0.399	0.383
2 Month	0.795	0.705	1	0.2507	0.225	0.270	0.273	0.050	0.059	0.070	0.015	0.149
4 Month	0.205	0.295	0.667	0.034	0.225	0.230	0.240	0.333	0.705	0.831	0.053	0.083
20 °C, t test pairing Day 0												
1 Month	0.070	0.126	0.023	0.225	0.423	0.075	0.079	0.478	0.029	0.024	0.000	0.384
2 Month	1	0.295	1	1	0.038	0.626	0.590	0.590	0.142	0.167	0.010	0.029
4 Month	0.228	0.278	0.667	0.456	0.808	0.823	0.844	0.323	0.374	0.382	0.909	0.070



Figures S5-1a and S5-1b. TS and VS (mg/L, Figure S1a) and *int*I1, *sul*1, and 16S rRNA gene copy numbers per milliliter digested sludge (gcn/µl, Figure S1b) for experimental triplicates of biosolids stored at 4 °C. Asterisks (*) denote P<0.05 in a statistical comparison Day 0 triplicates with a temperature/time triplicate.



Figures S5-2a and S5-2b. TS and VS (mg/L, Figure S2a) and *int*I1, *sul*1, and 16S rRNA gene copy numbers per milliliter digested sludge (gcn/µl, Figure S2b) for experimental triplicates of biosolids stored at 10 °C. Asterisks (*) denote P<0.05 in a statistical comparison Day 0 triplicates with a temperature/time triplicate.



Figures S5-3a and S5-3b. TS and VS (mg/L, Figure S1a) and *int*I1, *sul*1, and 16S rRNA gene copy numbers per milliliter digested sludge (gcn/µl, Figure S3b) for experimental triplicates of biosolids stored at 20 °C. Asterisks (*) denote P<0.05 in a statistical comparison Day 0 triplicates with a temperature/time triplicate.

CHAPTER 6: CONCLUDING REMARKS AND ENGINEERING SIGNIFICANCE

Wastewater treatment plants are critical control points between the environment and anthropogenic sources of pathogens, antibiotic resistant bacteria, and antibiotic resistance genes. Approximately 7.18 million tons of biosolids are generated, of which 3.95 million tons are applied to land each year (NEBRA, 2007) representing one of the largest opportunities for energy generation, resource recovery, and cost-effective (even profit-producing) sustainability in this country, but also one of the greatest challenges to protection of human and environmental health.

Recent accounts of adverse health impacts associated with exposure to land applied biosolids, coupled with studies observing an apparent resuscitation of pathogens and pathogen indicators and the widening realization of the importance of addressing environmental reservoirs of ARGs all lead toward the need for improved understanding of pathogen inactivation kinetics and mechanisms in sludge stabilization technologies.

This research has investigated the fate of two pathogens, MRSA and E. coli, and various ARGs under pasteurization, anaerobic digestion, biosolids storage, and land application conditions (Chapter 2). These studies taken as a whole suggest that a combination of analytical tools will be required to assess treatment efficiencies. Ambient and mesophilic temperature environments do not appear to induce the stress response associated with VBNC states, but pathogen die-off occurs at a rate specific to each pathogen and matrix. Furthermore, a lag in DNA degradation following cell death was observed suggesting that opportunities for transformation-related gene transfer exist even in the absence of viable cells. VBNC states are initiated by thermal treatments, such as thermophilic digestion and possibly pasteurization, and allow the persistence of pathogen cells and any ARGs contained therein through treatment and into the receiving environment where resuscitation or transformation could occur. Assessing cell death under thermal treatment conditions is difficult. Culturability is not an appropriate measure of VBNC cells and likewise, DNA quantification via QPCR is also lacking because QPCR detects live, dead, and VBNC DNA. Damage to cell membranes as measured by live/dead staining can be a transient state, reversed by subsequent repair and resuscitation. More work is needed in this area

to determine conditions that induce cell death, as well as, appropriate analytical tools to assess cell death.

These studies have shed some light on factors influencing the proliferation of ARGs in anaerobic digestion and biosolid storage. Results suggest that the ARG content of the raw sludge entering digestion does affect the effluent quality (Chapter 3), although the predominant mechanisms of ARG persistence may be different in mesophilic versus thermophilic digestion. In mesophilic digestion, the operating temperature of the digester allows a wider range of microbial diversity in the digester. As such, a higher percentage of bacteria in raw sludge may be able to survive the mesophilic digester environment and there may exist a greater degree of capability for horizontal gene transfer. Thermophilic digestion is characterized by high temperature and high ammonia concentrations, which have been shown to limit microbial diversity. As such, ARB survival and horizontal gene transfer was observed to increase in mesophilic digestion compared to thermophilic digestion (Chapter 3). Surprisingly, the experiments that introduced various concentrations of antibiotic sulfamethoxazole and antimicrobial nanosilver did not induce enhanced rates of horizontal gene transfer (Chapter 4). ARG concentrations in digesters receiving sulfamethoxazole concentrations above the minimum inhibitory concentration did not show elevated levels of ARGs to the unamended control. Similar results were observed with the nanosilver-amended digester; however, solubility calculations and a parallel study utilizing transmission electron microscopy (data not shown) showed that added nanosilver was quickly scavenged to produce silver sulfide precipitates, which lose their antimicrobial properties relative to nano- or ionic silver.

Finally, it was observed that ARG concentrations increased during 4 °C storage of raw sludge awaiting use in digester experiments. The implications of cold temperature exposure during winter storage of biosolids was investigated (Chapter 5). This study did show an elevation of ARG content in biosolids during short-term storage (less than two months); however, the levels returned to background within four months. The increase in ARG concentrations was more pronounced at lower temperatures than ambient temperatures, suggesting that there is a cold temperature stress induction of horizontal gene transfer of integron-associated ARGs. Thus, lengthy storage times (between two and four months) allow elevated ARGs to return to background levels.

The fate of ARGs is as complex as the microbial ecology and interactions in the engineered environment of sludge stabilization processes as a whole. ARG content of a digester cannot be disentangled from the fate of the ARG host, in particular in mesophilic digestion where a wider variety of microorganisms are able to assimilate, adjust, survive, or even thrive in the digester condition. High temperature conditions that limit microbial diversity in thermophilic digestion appear to reduce ARG content as a result. Survival and in particular, growth, of ARBs introduced into any treatment environment represent a reservoir of donor bacteria for future transfer and a reservoir of available ARGs for dissemination. In addition, experiments have shown the horizontal gene transfer likely occurs in mesophilic digestion, although the results are not as clear in thermophilic digestion.

Putting these results into action is merely speculation and brainstorming at this point. It is lofty to entertain the idea that seeding a thermophilic digester with ARG-free microorganisms could lead to an ARG-free effluent (biosolid). It seems plausible that due to the apparent limitations on microbial diversity and the resultant limitations on horizontal gene transfer, that thermophilic digestion could achieve a higher rate of ARG reductions compared to mesophilic digestion. However, it is evident that horizontal gene transfer still does occur, particularly with highly mobile integrons, so that complete reduction of all ARGs would not be possible with thermophilic digestion alone. More work is necessary to investigate pre-treatment options, such as thermal hydrolysis, that could significantly reduce ARBs prior to feeding to thermophilic digesters that are genetically engineered with minimal ARG content. The persistence and transformation capability of extracellular and VBNC DNA (ARGs) during and after thermal treatments will also influence any engineered solutions to reduce or eliminate antibiotic resistance during sludge stabilization processes.

References

Northeast Biosolids and Residuals Association (NEBRA). July 20, 2007. A National Biosolids Regulation, Quality, End Use, and Disposal Survey. Final Report.