Monitoring and Quantifying Tetracycline Resistance Genes in a Swine Waste Anaerobic Digester over a 100-Day Period

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MONITORING AND QUANTIFYING TETRACYCLINE RESISTANCE GENES IN A SWINE WASTE ANAEROBIC DIGESTER OVER A 100-DAY PERIOD

A Thesis
Presented to
The Faculty of the Department of Chemistry
Western Kentucky University
Bowling Green, Kentucky

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By
Melanie Couch

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MONITORING AND QUANTIFYING TETRACYCLINE RESISTANCE GENES IN A SWINE WASTE ANAEROBIC DIGESTER OVER A 100-DAY PERIOD

Date Recommended 3/23/18

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Dr. Kevin Williams

Dr. John Loughrin

Dean, Graduate Studies and Research Date 3/27/18
I dedicate this thesis to my sister whom we lost too soon, Mary Magdalene Eversole, who is a great inspiration to me.
ACKNOWLEDGEMENTS

“Alone we can do so little; together we can do so much.” --- Helen Keller

This work would not have been possible without the help of many others. I would first like to thank my advisor Dr. Eric Conte of the chemistry department at Western Kentucky University. Dr. Conte was always an open door or an email away whenever I ran into difficulties or had questions. He has allowed this thesis to be my own work but provided much needed guidance along the way.

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MONITORING AND QUANTIFYING TETRACYCLINE RESISTANCE GENES IN A SWINE WASTE ANAEROBIC DIGESTER OVER A 100-DAY PERIOD

Melanie Couch May 2018 83 Pages

Directed by: Dr. Eric Conte, Dr. Kevin Williams, and Dr. John Loughrin

Department of Chemistry Western Kentucky University

Unregulated use of growth promoting antibiotics like Tetracyclines in agricultural feeds is becoming an increasing problem in antibiotic resistance. Undigested antibiotics leads to significant concentrations in livestock waste. These concentrations provide continuous selection pressure for the development of antibiotic resistance genes in the environment. Antibiotic resistance related deaths are projected to surpass cancer related deaths by 2050 making antibiotic resistance a pressing public health issue. The purpose of this study is to determine the abundance and persistence of tetracycline (tet) resistance genes in swine waste over a period of 100 days in an anaerobic digester system. Tet(A), tet(B), tet(G), tet(M), tet(O), tet(Q), and tet(W) were quantified by quantitative polymerase chain reaction after DNA extraction. Primers that target ribosomal protection proteins and efflux proteins were used. Antibiotic resistance genes decreased from day one but were found to be present throughout the study.
1. Introduction

1.1 Background

Paul Ehrlich and Alexander Fleming are the pioneers of modern antibiotics.\(^1\) An important quality for antibiotics is the ability to selectively kill or inhibit microbial targets without causing harm to the human or animal host. Ehrlich realized the possibility of microbe selectivity while observing synthetic dyes. Aniline and other synthetic dyes would stain one microbe and not another. This observation led Ehrlich to begin a large-scale screening program in 1904 for a drug to treat syphilis, which was rampant and untreated at the time. One at time, compounds were treated for specificity and effectiveness until a suitable treatment was found. With the help of fellow scientist, Alfred Bertheim, Salvarsan was synthesized. Salvarsan successfully treated syphilis and was the most frequently prescribed drug until the discovery of penicillin in the 1940s. The effective systematic screening method for drug discovery using diseased live animal models was adapted by many scientists. For example, Bayer scientists used the method to discover sulfa drugs in 1935.

On September 3, 1928, Alexander Fleming observed the antibacterial properties of mold that led him to the discovery of penicillin. Alexander’s relentless determination brought awareness to the antibiotic’s potential while trying to get chemists to purify and stabilize the drug. In 1940, an Oxford led team published a paper on purification methods of penicillin which allowed for the mass production and distribution of the drug by 1945. Fleming’s screening method eliminated the need to use diseased live animal models, by instead using inhibition zones in lawns of disease causing bacteria grown on agar plates.
Fleming was also one of the first scientists to recognize and warn against antibiotic resistance.

The drug discovery methods of the pioneer antimicrobials, Salvarsan, sulfa drugs, and penicillin paved the way for future drug discovery and development. The years 1950 thru 1970 was considered the peak period for all antibiotic class discovery. Advancements in chemical synthesis lead to several classes of antibiotics, which are cataloged by their chemical structure and mechanism of action as seen in Figure 1. Antibiotic types number well into the hundreds, but there are only a few major classes. The major classes are penicillins, cephalosporins, macrolides, fluoroquinolones, sulfonamides, tetracyclines, and aminoglycosides. Many antibiotic classes inhibit RNA, DNA, cell wall synthesis, or protein production using only a few molecular targets within the bacteria. β-lactams and peptides such as penicillin and cephalosporin inhibit cell wall synthesis. Macrolides, tetracycline, fluoroquinolones, sulfonamides, and aminoglycosides inhibit protein production.

Figure 1 Antibiotic mechanism of action within the bacterial cell. Adapted from Ref. 5.
When antibiotics were first discovered, the likelihood of bacteria developing resistance was not considered, because the rate of mutations in bacteria was not considered significant. Bacterial horizontal or lateral transfer, the ability to interchange genes and/or plasmids, was also an unknown process that was later found to contribute to antibiotic resistance. In bacterial horizontal transfer, genetic information is transferred from one bacteria to another other than from parent to offspring.

β-lactams and aminoglycosides were the first antibiotics to encounter resistance. Since then, antibiotic resistance has been an increasing concern to public health. In the United States alone antibiotic resistance costs twenty billion dollars in health care annually. The true cost of antibiotic resistance will be the lives lost to simple bacterial infections when antibiotics are not effective. Antibiotics can be used either therapeutically or as a preventative measure. Furthermore, some antibiotics like tetracyclines can be used for animal growth promotion. The agriculture industry utilizes antibiotics like tetracycline in disease prevention and growth promotion. Consequently, this overuse of growth promoting antibiotics provides a continuous selection pressure in animal waste. Selection pressure is defined as any change in the environment that allows a certain mutation to survive and be passed on. Overuse and the absence of a variety of molecular targets for antibiotics to act upon plays a role in the development of antibiotic resistance. Antibiotic resistance is the bacterial development of a tolerance or resistance to the antibiotic over time.

1.2 Tetracyclines Class
The antibiotics in the tetracyclines class were first discovered in the 1940s as a broad-spectrum agent.\(^8\) Tetracyclines inhibit protein synthesis by blocking the binding of tRNA in the A position because of ribosomal binding interaction.\(^9,10\) Blocking the binding of tRNA prevents vital protein synthesis.\(^8\) Antibiotics in the Tetracycline class are both naturally occurring and synthesized molecules.\(^9\) Figure 2 depicts the structures of chlortetracycline and oxytetracycline, the first of the tetracycline class to be described.\(^11\) The linear fused ring system and hydrophilic functional groups are necessary for antimicrobial activity.\(^9\) Uptake and solubility are important because the molecule must transverse one or more membranes to get to the ribosome.\(^12\) Many substitutions to the linear fused ring system have been synthesized for optimization that has resulted in the variety of tetracyclines in clinical use today. Some of these substituents have increased solubility, which is vitally important for determining administration, delivery pathway, and toxicity of the antibiotic. Substituents on the linear fused ring system play a role in uptake and delivery by optimizing solubility.

1.3 Tetracyclines Use in Agriculture

High density population in livestock operations allow pathogens to spread easily and require aggressive infection management protocols.\(^13\) Three main uses of antibiotics
in agriculture are treatment of infections, prevention of diseases, and growth promotion. Antibiotics in the tetracyclines class such as tetracycline, oxytetracycline, and chlortetracycline are effective antimicrobials without major adverse side effects and as a result, this contributed to their popularity for agricultural animal husbandry.\textsuperscript{10} Tetracyclines are one of the few antibiotics available known for growth promotion, though the mechanism is unclear.\textsuperscript{14} Tetracyclines are among the most commonly used antibiotics in the agriculture industry. An estimated 30 to 70 percent of antibiotic use in the United States is utilized in livestock husbandry and meat production.\textsuperscript{15} These antibiotics are poorly absorbed in the gut by the animals and an estimated 40 to 90 percent are excreted in waste or urine in the parent or metabolized form.\textsuperscript{14}

1.4 Waste Management

A commonly used method of waste management on industrial farms is the flushing of solid and liquid waste into an open-air basin known as a lagoon.\textsuperscript{16} This poorly treated lagoon slurry is directly applied to soil as a crop fertilizer. Anaerobic digestion is another common method of livestock waste management, with an added goal of methane gas production for fuel.\textsuperscript{17} Overuse of antibiotics like tetracycline can create a continuous selection pressure for the development of bacterial resistant population in these waste management systems, with the potential of spreading to the environment.\textsuperscript{16} Of the few most common waste management systems, lagoons are the simplest. According to the American Society of Agricultural Engineers, lagoons are a waste treatment impoundment where water is added to manure to create a high degree of dilution for the primary goal of pollution reduction through biological activity. Microbial communities are segregated
and play a unique part in the degradation process within the treatment and sludge storage layers of the lagoon. Photosynthesizing bacteria reduces nitrogen and sulfur containing compounds and helps to eliminate odor in the effluent storage layer. Media and the public use the term “lagoon” as a blanket term for all open-air waste basins.

Biological processes play an integral role in the degradation of waste. Lagoon basins are known as cells, single-celled or multi-celled. Single-celled basins contain all the biological layers in one lagoon. Multi-celled basins have their biological functional layers split between the multiple cells. Figure 3 illustrates a single-celled lagoon and a multi-celled lagoon showing the segregated layers necessary for the digestion process. Photosynthesizing microbes are found in the effluent storage layer. The treatment layer is a gradient of aerobic and anaerobic bacteria. The sludge storage layer houses the settled solids and supports anaerobic digestion.

![Diagram of lagoon basins](image)

**Figure 3** (A) Diagram of a Single-cell lagoon illustrating one basin configuration. (B) multi-celled lagoon illustrating a split basin configuration. Adapted from Ref. 19.
Farm size is proportional to lagoon size and must provide storage for both effluent and sludge. The dilution must be maintained by adding influent to the lagoon sludge making sure not to reduce the volume of liquid beyond the minimum. Effluent is removed from the upper layers of the cell basins at a one to two foot depth and is often used as nutrients on crops without further treatment.

Lagoons may be covered or uncovered. Covering a lagoon may contribute to a higher level of anaerobic digestion than an uncovered lagoon because it is a closed system. Feeding the lagoon with organic material ensures all biological processes are continuous and provides nutrients and fresh microbes. Problems associated with liquid waste management include leakage, overflows, embankment failure, and odor emissions. Antibiotic contaminated waste in lagoons create an ideal environment for the development of antibiotic resistance genes by providing a continuous selection pressure. In fact, most lagoons are in direct contact with the ground with no barriers and thus these antibiotic resistance genes (ARGs) may enter the surrounding watershed.

Anaerobic digestion, much like lagoons, use biological microorganisms to break down organic matter but in the absence of oxygen. One goal of anaerobic digestion is biogas production through fermentation. Biogas consists of a mixture of methane, carbon dioxide, and trace amounts of other gasses, including water vapor. Biogas can be used to generate electricity or burned for heating and cooking. A properly constructed digester may reduce waste management costs, energy costs, bedding costs, and even generate revenue for farms. The two most common anaerobic digestion types are plug flow and complex mix. Seventy percent of operational farm digesters are either of these types. The
plug flow digester can handle from 11 to 13 percent solids; therefore, plug flow digesters are mainly used by the dairy industry because of the high density of cattle waste. Figure 4 illustrates the plug flow digester design. Plug flow digesters are designed so that the waste material (influent) goes into one end of an elongated tank and flows by optional stirring to the other end where it is removed as effluent. Biogas is captured by the tank’s covering and piped off to be used or sold. There is a constant flow as waste material is added and effluent is removed. Agitation may or may not be used for plug flow designs.

![Plug flow digester design](image)

**Figure 4 Plug flow digester design.** Influent port allows for the addition of waste. Effluent allows for removal of processed waste. Biogas is trapped by the lagoon dome.

Similarly, complex mixed digesters, such as the one in Figure 5, are supplied through a continuous flow of waste material but instead of an elongated tank, an upright central tank with consistent agitation is used. Complex digesters have a smaller footprint and are better suited for a higher liquid waste content than plug flow digesters but are slightly more expensive. Some complex digesters are located below ground to take advantage of constant ground temperatures. Because of the upright design of the complex digester, it can only handle from 3 to 10 percent solids and biogas is piped off as it is produced. Often the liquid waste is piped into the bottom of the tank with constant agitation to keep the waste material mixed. The waste that is piped in will move up the tank and through the microorganism rich layers to speed digestion. As waste is added to the tank, effluent is removed to prevent overflow and maintain correct dilution. Waste
material that is remaining after anaerobic digestion is separated into solid and liquid wastes. Solid waste can be used as animal bedding cutting the farmer’s cost. Furthermore, the material after digestion is less susceptible to bacterial growth because it has little to no organic material remaining. Liquid waste is either pumped back to a traditional storage facility like a lagoon or it is pumped onto crops. All the nitrogen that was present in the animal waste is converted by digestion to ammonia, which is a key ingredient in fertilizer.

![Diagram of a complex digester](image)

Figure 5 Simple illustration of a complex digester. Influent port allows for the addition of waste. Agitation moves waste from bottom to the top of the tank for removal. Effluent port allows for the removal of processed waste. Biogas pipe is for the removal of biogas as it is produced.

Both waste management lagoons and digesters provide a means of preparing waste for crop fertilizer and/or gas production. There are however uncertain effects with the presence of antibiotics like tetracycline on the antibiotic resistance gene population in these lagoons.\textsuperscript{20,21} It has been previously thought that digestion reduces the ARGs population. Newer studies suggest this may not be the case and tetracycline resistance genes are still present in high concentrations.\textsuperscript{22}
1.5 Antibiotic Resistance Genes

Bacteria have developed and passed on survival mechanisms against antibiotics by altering genetic information within the cell as mutations of genes or acquisition of foreign DNA coding resistance through horizontal gene transfer. Mutations in genes are developed by a subset of bacterial cells from the susceptible population. These mutated genes produce proteins that interfere with the antibiotics activity allowing the bacteria to survive. In general, resistance genes alter the activity of antibiotics via a few mechanisms, such as modification of the antibiotic, limiting antibiotic uptake into the cell, activation of efflux pumps to remove antibiotics from the cell quickly, or changes to the metabolic pathway targeted by the antibiotic. Therefore, resistance created as mutations in genes vary in complexity. The acquisition of foreign DNA through horizontal gene transfer is a prominent driver of bacterial evolution and is thought to be responsible for the dissemination of resistance to commonly used antibiotics. The most common and easily transferred mechanism is the acquisition of new genetic material by way of plasmids. Plasmids, as seen in figure 6, are an independent non-genomic mobile genetic element comprised of a small circular double stranded DNA unit located in the cytoplasm of a bacteria or protozoan.

Figure 6 illustration of bacterial genomic DNA and plasmids
Tetracycline resistance genes are located in these non-genomic plasmids. The three methods used by bacteria for gene transfer between cells, also known as horizontal transfer, are transformation, transduction, and conjugation. Figure 7 is a representation of the three methods of horizontal transfer. Bacteria that take up DNA from the environment use transformation. Transduction is the process by which bacteriophages move genetic material from one cell to the other. In conjugation, genes are transferred by way of plasmids from one unrelated cell to the other.

![Bacterial horizontal transfer](image)

Figure 7 Bacterial horizontal transfer by transformation, conjugation, and transduction. Adapted from Ref. 26.

Conjugation is the focus of previous tetracycline resistance research because the tetracycline resistance genes are in the plasmid element. As mentioned earlier, plasmids are separate from genomic DNA and replicate independently and do not code for basic cell function but instead codes for survival under certain selection pressures like a tetracycline contaminated waste. The over-use of tetracycline in agricultural industry leads to tetracycline being excreted in waste that accumulates in lagoons and anaerobic digesters. This creates optimal conditions for the development of tetracycline resistance genes. As a result, lagoons and anaerobic digesters become an overwhelming source for
tetracycline resistance genes in the environment. A cell can carry as few as one plasmid or as many as thousands. A resistance plasmid carries one or more antibiotic resistance genes. Quantifying plasmids are reported as copies per volume because a cell carries an unknown number of them. Quantification of antibiotic resistance genes is performed with quantitative real-time polymerase chain reaction (qPCR). Before explaining qPCR, tetracycline resistance genes will be discussed.

1.6 Tetracycline Resistance Genes

According to previous research, the majority of tetracycline resistance genes assayed were found in wastewater treatment plants which suggests that livestock waste is a major source of environmental tetracycline antibiotic resistance genes (ARGs). As of 2015, there have been over 40 classes of tetracycline resistance genes discovered in the environment. Currently, a resistance gene is assigned to a class based on DNA-DNA hybridization measured by melt temperature and each class is given a letter designation. Tetracycline efflux, ribosomal protection, and inactivation by enzymes are the three tetracycline resistance mechanisms identified in clinical isolates. Table 1 lists the more common tetracycline resistance genes found in animal waste lagoons and digesters. The efflux pump mechanism is a mutation in the sequence coding efflux pump membrane bound proteins that results in an over expression or over production of the protein. As a result, the antibiotic gets pumped out of the cell at an accelerated rate and the bacteria survive. Ribosomal protection proteins bind to the antibiotic’s target, in this case, the ribosome. This changes the antibiotics ability to bind to the ribosome, resulting in resistant bacteria. In Enzymatic inactivation, once the antibiotic enters the cell, enzymatic
proteins expressed from the ARG degrade or alter the antibiotic so it cannot be effective. A single bacterial cell can have one or more of these mechanisms active at any given time. The National Center for Biotechnology Information maintains a database that hosts the DNA sequences and characterizations for tetracycline resistance genes among others. Knowing the sequences of the DNA is very important for quantifying these genes using qPCR.

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Efflux</strong></td>
<td>tet(A), tet(B), tet(C), tet(D), tet(E),</td>
</tr>
<tr>
<td></td>
<td>tet(G), tet(H), tet(I), tet(J), tet(Z),</td>
</tr>
<tr>
<td></td>
<td>tet(30), tet(31), tet(K), tet(L), otr(B),</td>
</tr>
<tr>
<td></td>
<td>tcr(3), tetP(A), tet(V), tet(Y), tet(33),</td>
</tr>
<tr>
<td></td>
<td>tet(35), tet(38)</td>
</tr>
<tr>
<td><strong>Ribosomal Protection</strong></td>
<td>Tet(M), tet(O), tet(S), tet(W), tet(Q),</td>
</tr>
<tr>
<td></td>
<td>tet(T), otr(A), tetB(P)</td>
</tr>
<tr>
<td><strong>Enzymatic</strong></td>
<td>Tet(X)</td>
</tr>
</tbody>
</table>

### 1.7 Polymerase Chain Reaction

Polymerase chain reaction (PCR) target sequences of DNA or complementary DNA (cDNA) that can be copied many thousands of times using specific oligonucleotides, heat stable polymerases, and thermal cycling. In traditional endpoint PCR detection, samples are tested for either presence or absence of the target, requiring post reaction analysis using gel electrophoresis for quantification. In contrast, real-time quantitative PCR (qPCR) measurements are taken at each cycle of the amplification phase allowing the quantity of genes to be determined with great precision. Measurement at each cycle is performed with dyes, whose fluorescence intensity is directly proportional to the number of PCR products generated. These fluorescent dyes like...
SYBR green in Figure 8 are double-stranded DNA binding fluorescent dyes that anneal to the DNA during amplification.

![Polymethine structure of SYBR Green I](image)

Figure 8 Polymethine structure of SYBR Green I

The use of sequence specific primers allows the number of copies of target DNA or RNA sequence to be determined. Primers are short single stranded sequences used as a starting point for DNA synthesis. PCR reactions are done using about 20 µl a master mix buffer reagent solution, which includes the dye and 5 µl of template DNA solution. Master mixes can be made, bought premade, or sold in a kit that includes a heat stable polymerase, free deoxyribonucleotide triphosphates (dNTPs), a DNA binding fluorescent dye and forward / reverse primers. Standard curves are automatically created within the instrument using known concentrations of the target genes. Gene quantities are reported in copies per milliliter. 16S data is a quick and inexpensive way to determine the number of cells per volume in a sample. In bacteria 16S genes average four copies per cell. Using the data calculated from qPCR, a fairly accurate count of the total number of cells in the sample is obtained.
There are three major steps in PCR reactions; denaturation, annealing, and extension. Figure 9 demonstrates the general theory behind qPCR. Denaturation, Figure 9(A), occurs at a high temperature incubation which is used to denature or “melt” DNA into single strands. Complementary sequence primers hybridize during annealing to begin DNA synthesis (Figure 9B). Annealing temperature is based on the melting temperature of the primers and is usually about 5°C degrees below the denaturation temperature. Once the primers have annealed to the target single-stranded DNA, the next step is extension, Figure 9(C), which occurs in the range of 70 to 72°C. The activity of the DNA polymerase is optimal and primer extension occurs at a rate of just under 100 bases per second provided enough deoxyribonucleotide triphosphates (dNTPs) are present. Once primers anneal to the single strand of DNA fluorescence increases under blue light. The
longer the DNA sequence the higher the intensity. This is also true for the number of copies of DNA targets. The more copies made during the cycles the higher the intensity.

1.8 Research Proposal

The purpose of this research was to determine the effects of anaerobic digestion on the quantity of tetracycline resistance genes in swine waste over a period of one hundred days. Antibiotic resistance, a major threat to public human health, which limits effective treatment against infections and diseases. Therefore, an effective method for dealing with antibiotic contaminated waste is needed. Seven tetracycline resistance genes (\textit{tet}(A), \textit{tet}(B), \textit{tet}(G), \textit{tet}(M), \textit{tet}(O), \textit{tet}(Q), and \textit{tet}(W)) were measured in swine waste from a local Kentucky farm digested in two environment controlled 1000-liter anaerobic digester tanks in three separate trials. Quantities of tetracycline resistance genes were compared to 16S data. The Tetracycline class, commonly chlortetracycline and oxytetracycline, is popular in the agricultural industry and was chosen for this study. Various samples of digested waste were measured throughout the one hundred days to determine if anaerobic digestion reduced the number of the seven tetracycline resistance genes.

2. Experimental

2.1 Digester Design

Digester design was developed by John Loughrin at the U.S. Department of Agriculture, Agricultural Research Services, Food Animal Environmental Systems Research Unit (USDA-ARS, FAESRU) of Bowling Green, Kentucky before gene
research began as shown in Figure 10. This section as well as Digester Operation was adapted from the submitted publication Couch et al. Dual digesters were constructed from 1040 L (275 gallon as sold) blow-molded intermediate bulk container (IBC) tanks with a length of 1.2 m, width of 1.0 m and height of 1.15 m. A hole was drilled into the top of both tanks to house 1.27 cm diameter cross-linked polyethylene (PEX) pipe with an attached manual ball valve used as the waste and feed inlet. Float level switches (Omega Engineering Inc., Norwalk, CT) were installed in the side of the tanks to maintain the tank volume at 800 L. The float level switch was used to activate an electrical relay (American Zettler, Inc., Aliso Viejo, CA) routing power to a 1.27 cm full port solenoid-actuated 120-VAC PVC ball valve (Valworx, Inc., Cornelius, NC) installed on 1.27 cm diameter PVC pipe placed 44 cm above the tank bottom that served as the waste outlet (effluent).

The top of each IBC tank was adapted to accommodate a 3-way luer valve and 6.35 mm tubing that served as a gas outlet and sampling port. The tubing was connected to a Wet Tip Flow Meter® (wettipgasmeter.com) by one arm of a 3-way luer valve fitting. The other arm of the fitting accommodated a syringe for taking samples for gas analysis. The side of the tank had an addition 0.635 cm diameter port with 2-way luer valve installed 34 cm above the sludge for liquid analysis (digestate liquid). All pipe and tubing connections to the tanks were made with Uniseal® pipe to tank fittings (US Plastic, Inc.).
2.2 Digester Operation

Initial swine waste was obtained from a waste lagoon of a farrow to finish operation located in north-central Kentucky. Initially, 1,000 L of swine waste was pumped into each tank which activated the float switch controlled waste outlet as a means of partially concentrating wastewater solids and attaining the operating wastewater volume of 800 L.

The experiments were conducted in duplicate as three separate trials of 100 days each. In the first trial, the digesters were fed 290 g of a 2-parts ground corn to 1-part defatted soybean meal in 57 L of water twice weekly for a total of 8.41 kg. In the second trial the digesters were fed 565 g corn twice or three times weekly for a total of 23.84 kg, and in the third trial the digesters were fed 700 g corn meal, later increased to 1 kg of corn meal twice or three times weekly for a total of 62.5 kg. All feed was antibiotic free as attested by the vendor and confirmed by chemical analyses.
Gas production was measured daily during the workweek and averaged over the weekends. Gas and wastewater quality was measured weekly as described in Loughrin et al.\textsuperscript{29} Gas measurements ensured the health of the microbial community.

### 2.3 Sample Collection

Six different sample types were collected during digester feedings once a week on average during three different 100-day experiments. Samples were taken of the feed fed to the swine (\textit{feed}) and the corn mixture used to activate and feed the digesters (\textit{corn}). In addition, samples were taken of the initial waste collected from the farm (\textit{initial samples}), the overflow liquid as a result in tank feedings (\textit{effluent}), the liquid treatment microbial layer (\textit{digestate liquid}), and the settled solids sludge layer (\textit{sludge}). Samples were collected and stored at -20°C until processed.

### 2.4 Quantitative PCR

DNA was extracted from 500 µL of the liquid samples and 500 mg of the solid samples using FastDNA Spin Kit for Soil (MP Biomedical, Santa Ana, CA) following manufactures protocol. Extracted DNA solutions were frozen at -20°C for further analysis. Real time quantitative polymerase chain reaction (qPCR) was used to quantify gene concentrations from all bacterial cells (16S rRNA) using Qiagen HotStarTaq Master Mix (Qiagen, Valencia, CA) and seven tetracycline resistance genes (\textit{tet(A), tet(B), tet(G), tet(M), tet(O), tet(Q), tet(W)}) using QuantiTect SYBR green master mix (Qiagen, Valencia, CA). Primers used in qPCR assays were purchased from Integrated DNA Technologies (IDT, Coralville, Iowa) and 16S rRNA qPCR assay duel-labeled black hole quencher probes were purchased from Biosearch Technologies, Inc. (Petaluma, CA).
Primers and probes used were chosen from published protocols listed in Table 2.\textsuperscript{1,30–34} qPCR assays were performed according to Cook et al.\textsuperscript{35} with samples diluted in a 1:100 ratio to prevent PCR inhibitor effects. Dilution ratio was determined by performing qPCR assay on spiked samples to test inhibition at different dilution factors.

Table 2 Sequences, target sizes and melting temperature of primers used for quantitative real-time PCR for the quantification of tetracycline resistance genes (tet) under anaerobic digestion system. Reproduced from Couch et al.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer</th>
<th>Primer sequence (5'-3')\textsuperscript{†}</th>
<th>Tm (°C)\textsuperscript{‡}</th>
<th>PCR product (bp)\textsuperscript{§}</th>
<th>Assay type\textsuperscript{¶}</th>
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Melt curves were done on each assay run at a range of 65°C to 95°C at 0.2°C intervals. Melt curves ensures one product formation during PCR assays. qPCR assays were performed using Bio-Rad CFX96 real time PCR detection system (Bio-Rad, Hercules, CA). Of the primers found in previous literature all were efficient except primers used for tet(M). Many trial qPCR assays needed to be run to determine the optimum primer set used.

2.5 Primer Optimization

qPCR accuracy depends on primer optimization. Optimization is necessary for reproducible results with the desired sensitivity and specificity. The primers used for qPCR quantification in this research were optimized for efficiency with a standard curve, concentration, and primer-dimer potential. Primer-dimers occur when complimentary primers anneal together instead of the target sequence. Standard curve efficiency is a measure of amplification rate expressed as a percentage. Ideally, amplification should double the number of target molecules per qPCR cycle. Secondary structures, primer-dimers or hair pins, and improper annealing temperatures are common causes of poor efficiencies. Primer sets performing at / or greater than 85% efficiency for standard curve assay did not necessitate further concentration optimization.

Each primer set (forward and reverse) was chosen from previous research, therefore most of the optimization was already performed. All eight primer sets (tet(A),

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tet(B), tet(G), tet(M), tet(O), tet(Q), and tet(W) and 16S primer / probe set) used performed above 85% efficiency. Unchanged primer sets (forward and reverse primers from the same source) required optimization for the USDA qPCR instruments. For example, tet(A) used both forward and reverse primers from Ng et al31 research group and therefore a simple annealing temperature gradient was performed spanning 55°C to 65°C that included three known standard concentrations, two unknown, and negative samples. Table 3 shows the temperature gradient indicating all temperature results in a fluorescence detection cycle (Cq) within an expected standard range. Starting quantities (SQ) of the standards were used to calculate the SQ concentration of the unknowns.

Table 3 qPCR tet(A) temperature gradient assay. Temperatures ranging from 55°C to 65°C using standards 1 x 101 ng/µL, 1 x 103 ng/µL, and 1 x 105 ng/µL and unknown samples.

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<th>Fluor</th>
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<th>Sample</th>
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<th>SQ</th>
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<td>Std-2</td>
<td>1e3 tetA New Std</td>
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</table>
The amplification plot also indicates that all annealing temperatures produce a Cq in the expected location in Figure 11. The amplification plot is a graph of relative fluorescence units (RFU) versus cycle number. The lower the Cq number the higher the concentration of the initial sample. In other words, the higher the initial concentration the sooner fluorescence is detected.

Figure 11 Amplification plot for tet(A) standards 1x101 ng/µL, 1x103 ng/µL, and 1x105 ng/µL and unknown samples over a temperature gradient from 55°C to 65°C

The next step was to determine which temperature is optimal for tet(A) assay. The standard curve efficiency was examined keeping in mind higher annealing temperatures are preferred to prevent nonspecific binding. Primer efficiency is calculated using formula 1.37

\[
Primer\ \textit{efficiency} = 10^{\left(\frac{-1}{slope}\right)} - 1
\]

The ideal primer efficiency would be 100%, meaning the number of target molecules exactly doubles with each cycle. Efficiencies over 100% indicate polymerase
inhibitors, pipetting errors, reverse transcriptase, inaccurate dilution series, unspecific products, and primer dimers.

While excluding standard curves with efficiencies below 85%, the top three annealing temperature standard curves were considered, Figure 12. The 65°C assay resulted in a standard curve efficiency of 91.1%. The 64.5°C assay resulted in 100.8% efficiency and the 57°C assay resulted in 93.1% efficiency.

Figure 12 Standard curves from the temperature gradient assay for tet(A), A) is at 65°C, B) is at 64.5°C, C) is at 57°C.

The 57°C annealing temperature was chosen for further tet(A) assays. This temperature provided the highest acceptable efficiency. The 64.5°C assay was not considered because the efficiency was above 100%.

Melt curves were monitored to confirm only one product was amplified during the assay and no primer-dimers or nonspecific binding occurred. A primer-dimer is formed when primers anneal to each other because of complementary sequences in the primers. Melt curves are a plot of relative fluorescence units versus temperature. The qPCR instrument performs a melt curve assay over a range of temperatures and measures fluorescence. When SYBR green is used, the ideal melt curve should show an initial high fluorescence and drop as the temperatures increase and DNA becomes denatured as shown in Figure 13.
This is because SYBR green dye is a double stranded DNA binding dye. To get a better picture of the melting point of your target, 50% of the DNA is denatured, the first derivative of the melt curve is generated by the PCR instrument as illustrated in Figure 14. Peaks that appear before 78°C are more likely primer-dimers or due to nonspecific binding. The first derivative of tet(A) melt curve has a single peak that is higher than 78°C which indicates the melting point of the tet(A) gene is about 86°C. In addition, this indicates the primers are specific to the tet(A) gene with no primer-dimers. The tet(A) primer set optimum conditions were 57°C with 93.1% efficiency with one melt peak above 78°C. Standard curve efficiency assays were performed on each primer set using this method.

Figure 13 tet(A) melt curve from a primer temperature gradient test assay demonstrating the drop of fluorescence as temperature increases.
The *tet*(M) primer set required more optimization. First, the same annealing temperature gradient using Aminov et al\(^39\) primer set was performed spanning 55°C to 65°C including three known standard concentrations, two unknown, and negative control samples. Figure 15 is the results of the temperature gradient assay. Both plots indicate a failure and cannot be interpreted. The assay was repeated once more with the same results.

As a result, the next assay used the *tet*(M) Ng et al\(^31\) primer set with no temperature gradient. This *tet*(M) assay used a full standard set in duplicate at 59°C with

![Melt Peak](image1)

![Amplification](image2)

**Figure 14** First derivative of the *tet*(A) melt curve showing the melting point of the *tet*(A) gene.

**Figure 15** Amplification plot and Melt Peak plot for the temperature gradient for *tet*(M) using Aminov et al primer set.
no unknown samples. The results of this assay are seen in Figure 16. At first glance, the amplification plot looked good and the efficiency was just over the range at 113.2%. Unfortunately, the Cq occurred at a lower cycle number upon further examination. This could suggest that there are more copies per milliliter than measured by NanoDrop, contamination, or the size of the product was too large for an accurate measurement.

Figure 16 tet(M) Ng et al primer set standard test at 59°C with a full set of standards.

The next assay performed for tet(M) was using primers from two different literature sources. Forward primer was from Florez et al\textsuperscript{40}, and reverse primer was from Aminov et al\textsuperscript{39} research. Mixed matched primer sets (forward and reverse primers matched from different research articles) needed further optimization. Again, the reaction conditions were a full standard set in duplicate at 59°C with no unknown samples. The amplification plot, Figure 17, appeared to be within the range of detection but the actual Cq values told a different story.
Figure 17 Amplification plot for tet(M) assay using Florez et al forward primer and Aminov et al reverse primer at 59°C.

The results are recorded in Table 4. While the detection cycle for the samples that were detected occurred at an acceptable Cq, detection was below detection limits for the lower standard concentrations. A standard curve should show regular decreasing intervals between Cq as the concentration increases.

Table 4 tet(M) aPCR reaction using Florez et al forward primer and Aminov et al reverse primer at 59°C showing no detection for some of the dilute standards.

<table>
<thead>
<tr>
<th>Well</th>
<th>Fluor</th>
<th>Content</th>
<th>Sample</th>
<th>Cq</th>
<th>SQ (Number of copies)</th>
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<tr>
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<td>SYBR</td>
<td>Std-09</td>
<td>1e1 tetM Std Flor/Ami</td>
<td>N/A</td>
<td>1.00E+01</td>
</tr>
<tr>
<td>C02</td>
<td>SYBR</td>
<td>Std-10</td>
<td>1e2 tetM Std Flor/Ami</td>
<td>N/A</td>
<td>1.00E+02</td>
</tr>
<tr>
<td>C03</td>
<td>SYBR</td>
<td>Std-11</td>
<td>1e3 tetM Std Flor/Ami</td>
<td>N/A</td>
<td>1.00E+03</td>
</tr>
<tr>
<td>C04</td>
<td>SYBR</td>
<td>Std-12</td>
<td>1e4 tetM Std Flor/Ami</td>
<td>N/A</td>
<td>1.00E+04</td>
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<tr>
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<td>36.68</td>
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<td>SYBR</td>
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<td>1.00E+07</td>
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<td>SYBR</td>
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<tr>
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<td>N/A</td>
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<td>1e1 tetM Std Flor/Ami</td>
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<td>1.00E+01</td>
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<td>D02</td>
<td>SYBR</td>
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<td>1e2 tetM Std Flor/Ami</td>
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<td>1.00E+02</td>
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<td>1e4 tetM Std Flor/Ami</td>
<td>N/A</td>
<td>1.00E+04</td>
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<td>1e5 tetM Std Flor/Ami</td>
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<tr>
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<td>SYBR</td>
<td>Neg Ctrl</td>
<td>neg</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

More combinations of primers and annealing temperatures were assayed before moving to the next step. Another literature search resulted in the Vikram et al\textsuperscript{34} primer set that performed within efficiency range and had expected Cq values. Furthermore, the melt curve and melt peak confirmed one product was amplified.

2.6 Statistical Analysis

PCR data was converted to concentrations of copies of gene per mL for liquid samples and copies of gene per mg for solid samples. The qPCR instrument reports the quantification cycle (Cq) and starting quantity (SQ) of each sample assayed. The SQ was used to calculate the number of copies per PCR in the initial sample by dividing the starting quantity of the PCR reaction by the dilution factor, equation 2.
\[
(2) \quad \text{copies per PCR} = \frac{\text{SQ}}{\text{dilution factor}}
\]

The copies per PCR was used to calculate the number of copies per DNA elution. Copies per PCR is divided by the volume of DNA eluted by DNA extraction. Equation 3 is used to calculate copies per DNA elution. DNA elution volume was 100 µL.

\[
(3) \quad \text{copies per DNA elution} = \frac{\text{copies per PCR}}{\text{DNA elution volume}}
\]

Copies per DNA elution was then converted to copies of gene per mL or mg (gene concentration) using equation 4. Copies per DNA elution was divided by initial volume or mass of the sample used for DNA extraction. Initial volume was 500 µL and initial mass was 500 mg.

\[
(4) \quad \text{copies per mL or g} = \frac{\text{copies per DNA elution}}{\text{Initial sample volume}}
\]

Gene concentrations were then converted to \(\log_{10}\) (number of gene copies/unit of sample + 1) to account for large values in the data. Gene concentrations were averaged from the two digesters because they were not significantly different. All analyses were performed using Stata/SE 15 (Stata Corp. College Station, TX).

3. Results

3.1 Distribution of Tetracycline Resistance Genes

Mean concentrations of the genes encoding for total bacteria and tetracycline resistance are shown in Table 5. A single bulk animal feed sample was analyzed for 16S rRNA and \(tet\) genes. The concentrations of the 16S rRNA, \(tet(M)\), \(tet(Q)\), and \(tet(W)\) in the bulk swine feed sample were 9.41, 5.78, 4.70, and 4.33, respectively. The remaining
four *tet* genes (A, B, G and O) were not detected from the bulk animal feed sample. The concentrations of total bacteria (16S rRNA), *tet*(M), and *tet*(G) measured were not significantly different for all-sample types [p>0.05].

Table 5 Concentration of gene copies for total bacteria (16S rRNA) and tetracycline resistance genes (*tet*) averaged across the sampling days and two bio-digesters adjusted for the random effect of three independent trials. Results are presented as mean values of log₁₀ (gene copies/sample +1). Mean values with different letters in the column are significantly different (p<0.05) for each matrix-gene combination. Adapted from Couch et al.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>16S rRNA</th>
<th>tetA</th>
<th>tetB</th>
<th>tetG</th>
<th>tetM</th>
<th>tetO</th>
<th>tetQ</th>
<th>tetW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swine feed</td>
<td>9.12&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>BLD&lt;sup&gt;D&lt;/sup&gt;</td>
<td>BDL&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>BDL&lt;sup&gt;A&lt;/sup&gt;</td>
<td>5.57&lt;sup&gt;A&lt;/sup&gt;</td>
<td>BDL&lt;sup&gt;D&lt;/sup&gt;</td>
<td>4.17&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>4.04&lt;sup&gt;AB&lt;/sup&gt;</td>
</tr>
<tr>
<td>Corn</td>
<td>8.81&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>4.51&lt;sup&gt;A&lt;/sup&gt;</td>
<td>5.71&lt;sup&gt;B&lt;/sup&gt;</td>
<td>4.48&lt;sup&gt;ABC&lt;/sup&gt;</td>
<td>5.17&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.90&lt;sup&gt;D&lt;/sup&gt;</td>
<td>3.71&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.39&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Initial waste</td>
<td>9.19&lt;sup&gt;B&lt;/sup&gt;</td>
<td>6.45&lt;sup&gt;C&lt;/sup&gt;</td>
<td>5.39&lt;sup&gt;B&lt;/sup&gt;</td>
<td>6.25&lt;sup&gt;C&lt;/sup&gt;</td>
<td>5.84&lt;sup&gt;A&lt;/sup&gt;</td>
<td>7.07&lt;sup&gt;B&lt;/sup&gt;</td>
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<td>4.70&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.26&lt;sup&gt;A&lt;/sup&gt;</td>
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<td>6.99&lt;sup&gt;CD&lt;/sup&gt;</td>
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<td>Sludge</td>
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<td>6.20&lt;sup&gt;BC&lt;/sup&gt;</td>
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<td>5.33&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>6.86&lt;sup&gt;A&lt;/sup&gt;</td>
<td>7.04&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>8.26&lt;sup&gt;C&lt;/sup&gt;</td>
<td>7.69&lt;sup&gt;DE&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Where detectable, concentrations of *tet*(A), *tet*(O), *tet*(Q) and *tet*(W) in the bulk swine feed sample were not significantly different than those observed in the corn, effluent and digestate liquid samples [p>0.05], but significantly lower than that of the initial swine waste and sludge [p<0.05]. The concentration of *tet*(B) in the corn samples were not significantly different than that of initial swine waste lagoon and sludge samples [p>0.05].

Concentrations of total bacteria and the *tet* genes did not significantly differ between the effluent and digestate liquid samples which were obtained during the
digestion process \([p>0.05]\). In contrast, reductions were observed when comparing the sample types to the initial swine waste lagoon samples \([p<0.05]\). There was a 1.0 \(\log_{10}\) reduction of total bacteria in the effluent and digestate liquid samples which can be attributed to anaerobic digestion. We observed a 1.75 \(\log_{10}\), and a 1.23 \(\log_{10}\) reduction of \textit{tet}(A) concentrations in the effluent and digestate liquid samples, respectively. Even more evident is the reduction of \textit{tet}(B) by 3.1 \(\log_{10}\) in the effluent samples and by 2.3 \(\log_{10}\) in the port samples. The concentration of \textit{tet}(G) was reduced by 2.5 \(\log_{10}\) in the effluent samples and by 2.0 \(\log_{10}\) in the digestate liquid samples. For \textit{tet}(O), statistically significant reduction by 1.2 \(\log_{10}\) was observed in the effluent only. The concentration of \textit{tet}(Q) was significantly reduced by 1.4 \(\log_{10}\) in the effluent and by 1.1 \(\log_{10}\) in the digestate liquid samples. The concentration of \textit{tet}(W) was reduced by 1.2 \(\log_{10}\) in the effluent and by 0.83 \(\log_{10}\) in the digestate liquid samples. The sludge samples did not significantly differ with respect to the concentrations of total bacteria, \textit{tet}(A), \textit{tet}(B), \textit{tet}(G), \textit{tet}(M) and \textit{tet}(O) from other sample types \([p>0.05]\) except \textit{tet}(Q) and \textit{tet}(W) showed an increase compared to effluent and digestate liquid samples \([p<0.05]\).

The mixed effects model indicated there were significant differences between trials for both the total bacteria and \textit{tet} gene concentrations. To determine which concentrations varied by trial, a univariate analysis was performed using only trial as a factor for each gene in each sample type. All initial waste samples except for \textit{tet}(M) \([p=0.28]\) and \textit{tet}(O) \([p=0.25]\) were significantly different \([p<0.001]\). Corn samples were significantly higher in trial two than one except for \textit{tet}(B) \([p=0.74]\). No corn samples were analyzed in trial three. Effluent samples showed no significant difference over all the trials \([p>0.05]\). Digestate samples were not significantly different over the three trials.
except for \textit{tet}(A) \([p=0.03]\), \textit{tet}(B) \([p=0.008]\), \textit{tet}(M) \([p=0.003]\). Sludge samples except 16S rRNA \([p=0.04]\) and \textit{tet}(G) \([p=0.03]\) were not significantly different between trial two and trial three. No sludge samples were analyzed for trial one.

To determine if sampling day had an effect, data was analyzed by trial shown in Table 6. In trial one, corn sample concentrations for 16S rRNA \([p=0.69]\), \textit{tet}(A) \([p=0.59]\), \textit{tet}(B) \([p=0.86]\), \textit{tet}(G) \([p=0.75]\) were not significantly different by sampling day. However, \textit{tet}(M) \([p=0.03]\), \textit{tet}(O) \([p=0.01]\), \textit{tet}(Q) \([p=0.02]\), \textit{tet}(W) \([p=0.02]\) were significantly different by day for the corn samples. Effluent samples were only collected for day seven and no sludge samples were analyzed for trial one. Digestate samples did not significantly differ by sampling day \([p>0.05]\). In trial two the all sample types did not significantly differ by sampling day \([p>0.05]\). In trial three, only the effluent samples indicated a significant difference for 16S rRNA \([p=0.002]\), \textit{tet}(A) \([p=0.03]\), and \textit{tet}(O) \([p=0.005]\). All other gene concentrations were not significantly different \([p>0.05]\). Also, digestate and sludge samples did not differ significantly by sampling day in trial three.

Table 6 Mean gene copies of total bacteria (16S rRNA) and seven tetracycline resistance genes (tet) from various sample types collected during anaerobic digestion in three trials Mean values with different letters in the column for each matrix-gene combinations are significantly different at the 5% level. If letters are not shown for the matrix-gene combinations they did not differ by day. Results are presented as mean values of the log_{10} (gene copies/sample unit + 1). Reproduced from Couch et al.

<table>
<thead>
<tr>
<th>Sample matrix</th>
<th>Day</th>
<th>16S rRNA</th>
<th>\textit{tet}A</th>
<th>\textit{tet}B</th>
<th>\textit{tet}G</th>
<th>\textit{tet}M</th>
<th>\textit{tet}O</th>
<th>\textit{tet}Q</th>
<th>\textit{tet}W</th>
</tr>
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<td>5.64</td>
<td>4.30</td>
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<td>4.56</td>
<td>6.09</td>
<td>7.29</td>
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Trial 2

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**3.2 Tetracycline Antibiotic Concentration**

The tetracycline antibiotic levels were analyzed by other members of Dr. Conte’s research group that include, Dr. John Kasumba, Ali J. Abdulraheem, Christopher Fullington, and Courtney Cruse. The samples were extracted using weak cation exchange...
polymeric solid phase extraction (SPE) cartridges and analyzed for the target
tetracyclines by LC-MS/MS. Detection was done with two injections because the LC-MS
could not detect all 6 tetracyclines in a single run. The concentrations of three
tetracycline antibiotics, tetracycline (TC), chlortetracycline (CTC), and oxytetracycline
(OTC) and the main metabolites of tetracycline (4-epitetracycline (ETC)) and
chlortetracycline (iso-chlortetracycline (ICTC)) were measure in 57 samples collected
from the digester.

There were no discernible trends in the concentrations of TC and CTC over time.
OTC was not detected in the digester samples. TC was present in 68% and CTC was
present in 87% of the 57 extracted samples. TC ranged from below detection limits
(BDL) to 36 ppb in trial 1, BDL to 14 ppb in trial 2, and BDL to 31 ppb in trial 3. CTC
measure BDL to 112 ppb, BDL to 103 ppb, and BDL for trial 1, trial 2, and trial 3
respectively. Of the metabolite studies, ICTC was the most abundant measuring 0.37 to
11.9 ppm in trial 1, 0.54 to 2.85 ppm in trial 2, and BDL to 103 ppb in trial 3. ETC
concentrations ranged from BDL to 30 ppb in trial 1, BDL to 7 ppb in trial 2, and BDL to
19 ppb in trial 3.

3.3 Gas Production

The concentrations of carbon dioxide and, methane, chemical oxygen demand,
and pH in the digesters varied greatly among the three separate trials as shown in Table 7.
The average daily gas production was affected by feeding rates but gas quality was
similar averaging 21 percent carbon dioxide of the carbon dioxide / methane mixture.
Daily gas production for trial two and trial three was four and ten times that of trial one,
respectively. Trial one was fed nearly seven and half times as much feed that maybe
attributed to the drastic increase of gas production. Trial two and three’s environmental conditions were more favorable than trial one. The pH measurements in trials two and three are closer to biological pH than trial one due to bicarbonate buffering. A stable biological pH favors stable digestion and higher gas production. Chemical oxygen demand concentrations were about three-fold higher in trial three than in trial one and only slightly higher in trial two. Regardless, gas production was indicative of an active anaerobic digestion process producing methane averaging almost 80 percent in all three trials.

Table 7 a Data represent the mean ± standard error of the mean. Gas production measured daily and averaged over the weekend. Biogas and wastewater quality means represent the mean ± standard error of the mean of 32 determinations for trials 1 and 2, and 28 determinations for trial 3. Reproduced from Couch et al.

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<td>Carbon dioxide (µmole L⁻¹)</td>
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<td>Methane (µmole L⁻¹)</td>
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<td>pH</td>
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<td>Chemical oxygen demand (mg L⁻¹)</td>
<td>1,180 ± 141</td>
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<td>Bicarbonate buffering (µmole L⁻¹)</td>
<td>11.9 ± 1.4</td>
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4. Conclusion

Antibiotic resistance was not considered when antibiotics were first discovered.² Today we know antibiotic resistant bacteria are an increasing threat to public health.⁴ Research points to waste management systems in agricultural industry being a consistent
source of tetracycline resistance genes in the environment. In this study, the effects of anaerobic digestion on tetracycline resistance genes in swine waste were investigated. Two sample types were assayed during digestion (effluent and digestate) and one after the digesters were disassembled (sludge). The measured concentrations of these samples were compared to initial waste from the local swine farm’s lagoon. Tetracycline and tetracycline metabolite concentrations were also measured in various samples from the digesters as a measure of selection pressure. Gas production and water quality were monitored to ensure active digestion.

No observable difference in total bacteria concentrations (16S rRNA) were measured between effluent and digestate samples. The 100-day anaerobic digestion of swine waste resulted in statistically significant but not a significant biological reduction (1 – 2.5 log_{10}/ml) in tetracycline resistance gene concentrations in liquid and solid samples compared to initial waste samples. No additional tetracycline antibiotics were added during digester feedings after the initial waste to seed the tanks. Antibiotic free corn was used for tank feedings. The tetracycline resistance genes persisted even without added selection pressure. Anaerobic digestion is inefficient to reduce the spread of antibiotic resistance genes in the environment.

There were no obvious trends in tetracycline and tetracycline metabolites concentration over the 100-day digestion period. However, the concentration of ICTC showed an expected trend with an initial increase and later decrease in concentration for trial 2. This is expected because ICTC is a metabolite for CTC. As CTC degrades to ICTC, the concentration of ICTC will increase initially. The lack of trend could be attributed to a limited number of samples and many samples being below detection limits.
Despite the concentrations being sub-inhibitory for bacterial growth, they provide a selection pressure in the anaerobic digesters.

In conclusion, Anaerobic digestion of lagoon swine waste over 100-days did not biologically significant reduce tetracycline resistance genes concentrations. Considering the digesters were only fed antibiotic free corn, the antibiotic resistance genes were present in initial lagoon waste. The question remains whether the genes are a normal part of the farm microbial population or are acquired because of the use of antibiotics in feeds. More studies are needed to compare farms that use antibiotic feed / treatment and organic farms that do not use antibiotics. The agricultural industry and biochemists could collaborate to develop a waste management system to both improve gas production and reduce antibiotic resistance genes.

Waste conditions, pH level and temperature, play a role in bacterial survival. Considering sludge and effluent uses, existing waste management systems could be modified or new waste management systems could be developed to vary these conditions. A change in pH or increase in temperature degrades bacteria and DNA. To determine the effects of changes in pH or temperature, studies may be repeated using the same parameters and varying these conditions (pH and temperature).

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(38) Sigma Aldrich. "Optimizing qPCR."


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Appendix

Effect of anaerobic digestion of swine waste on the concentrations of tetracycline resistance genes and tetracycline antibiotics

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Keywords: Tetracyclines, Tetracycline Resistance Genes, Swine Waste, Anaerobic Digestion

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ABSTRACT

Anaerobic digestion produces biogas and digested material from animal waste. Its effect on antibiotic resistance genes (ARGs) and antibiotics is not widely studied. We investigated the effect of anaerobic digestion on seven tetracycline resistance (Tet') genes (tetA, B, G, M, O, Q and W), three tetracyclines (tetracycline, oxytetracycline and chlortetracycline), and two metabolites (4-epitetracycline and isochlortetracycline). Two identical 800-liter digesters were seeded with sludge from a swine waste lagoon and supplied antibiotic free feed in three separate 100 day trials. Tet' genes were measured by qPCR from total microbial community DNA extracted from initial swine waste lagoon, swine feed, corn mix, and digester samples (effluent, digestate liquid and sludge). Tetracyclines and their metabolites were extracted by solid phase extraction method and measured by LC-MS/MS. Concentrations of total bacteria and 6 tet genes (except tetM) in the effluent and digestate liquid samples showed significant reduction that ranged from 1 - 2.5 log_{10} reduction from their starting concentrations in the initial swine wastes. Concentrations of total bacteria and the tet genes in the sludge samples did not differ from their starting concentrations in the initial waste samples. Similar concentrations of total bacteria and tetracycline resistance genes could be detected from swine feed and corn samples. Up to 36 ppb (parts per billion; tetracycline), 112 ppb (chlortetracycline), 103 ppb (isochlortetracycline) and 30 ppb (4-epitetracycline) were detected; oxytetracycline was not detected. In conclusion, anaerobic digestion of animal waste has little to no effect in complete removal of bacteria, ARGs and antibiotics. Feeds used for animals and activation of anaerobic digestion can be a source of ARGs during the biodigestion process.
INTRODUCTION

Tetracyclines are broad-spectrum antibiotics used in animal agriculture for the treatment and prevention of diseases as well as growth promotion.\textsuperscript{46-51} In the United States and China alone, the combined annual production of antibiotics has reached 276,000 tons of which 48% is used in agriculture.\textsuperscript{50-52} Tetracycline class of antibiotics (tetracycline (TC), chlortetracycline (CTC) and oxytetracycline (OTC)) are the most commonly used antibiotics in livestock production in the United States\textsuperscript{53,54} and other parts of the world.\textsuperscript{14,50,55,56} In the United States tetracycline is classified as a medically important class of antibiotics based on its importance for human use.\textsuperscript{57} The U.S. Food and Drug Administration (FDA) summary report indicates that 5,866.6 metric tons of tetracyclines were sold in 2016 for use in animals accounting for 70% of the medically important classes of antimicrobials, and 42% of all antimicrobial sales for animal use in the United States.\textsuperscript{54} In the same time period 18% of all antimicrobials and 43% of tetracyclines sold and distributed for use in food animal production were sold for use in swine. For the purpose of growth promotion, antibiotics are usually added to animal feed resulting in overuse due to the lack of regulations particularly in developing countries\textsuperscript{51,58}, a practice that has been banned in European Union since 2006\textsuperscript{59}; and the use of medically important antibiotics for growth promotion purposes has been eliminated in the United States as of January 1\textsuperscript{st}, 2017 in alignment with Guidance for Industry (GFI #213) document.\textsuperscript{57}

Antibiotics and their metabolites are excreted from the animal body through feces and urine.\textsuperscript{49,60,61} As a result, levels of bacterial antibiotic resistance (AR) increase in animal farm waste lagoons and in the surrounding environment.\textsuperscript{51,62} Antibiotic resistance is a pressing issue of public health. Antibiotic resistant genes (ARGs) can render human
antibiotics ineffective, limiting the treatment of infections and lead to the emergence of “superbugs” - bacteria that are resistant to almost all currently available antibiotics. This has become a serious threat resulting in at least 23,000 deaths each year in the United States alone. Understanding the lifespan of excreted antibiotics such as the tetracyclines and their associated resistance genes (RGs) is the first step of reducing the spread of antibiotic resistance.

Tetracycline resistance is manifested via efflux proteins, ribosomal protection proteins, inactivating or degrading enzymes, or through unknown mechanisms. Efflux protein genes belong to the major facilitator superfamily (MFS) and code for membrane associated proteins that export tetracyclines out of the cell to reduce the concentration of the antibiotics within the cell. Ribosomal protection proteins interact with the ribosomes, reducing the tetracycline’s effectiveness. Forty-six different tetracycline resistance genes have been reported consisting of 30 efflux proteins, 12 ribosomal protection proteins, three inactivating proteins, and one unknown mechanism.

Anaerobic digestion is a common method of reducing animal waste and provides useful biogas. The goal of an anaerobic digestion system is to improve the effluent quality while creating green energy. Anaerobic digestion is not only widely used to treat swine wastewater for the degradation of organic matter, but it may be considered an effective method of reducing ARGs in livestock waste. Studies have reported decreases in ARGs during the anaerobic digestion of human wastewater and animal manure. Very limited literature is available on the fate of tetracyclines in swine manure during anaerobic digestion, while a number of studies have investigated the fate of tetracyclines during swine manure composting.
The main objective of this study was to determine the effect of anaerobic digestion on the concentrations of tetracyclines and tetracycline resistance genes over a three-month period. The concentrations of three tetracycline antibiotics (TC, CTC, and OTC) and the main metabolites tetracycline (4-epitetracycline) and chlortetracycline (iso-chlortetracycline), and seven tetracycline resistance genes (hereafter referred to as tet genes: tetA, tetB, tetG, tetM, tetO, tetQ and tetW) in swine waste obtained from a local Kentucky farm were measured over a period of approximately 100 days in two identically controlled 800-liter anaerobic digesters in three separate trials. We chose tetracyclines for this study because tetracyclines (mainly chlortetracycline and oxytetracycline) are the most commonly used in-feed antibiotics in swine production in the United States53. Furthermore, tetracycline resistance is also the most widespread in enteric bacteria of swine origin.77,78

MATERIALS AND METHODS

Dигester Design

Duplicate digesters were constructed from 1040 L (275 gallons as sold) blow-molded intermediate bulk container (IBC) tanks with a length of 1.2 m, width of 1.0 m and height of 1.15 m. The top of each tank had a hole drilled into it to accommodate 1.27 cm diameter cross-linked polyethylene tubing fitted with a manual ball valve that served as the waste inlet. This pipe extended into the tank below the surface of the digestate liquid. Float level switches (Omega Engineering Inc., Norwalk, CT) were installed in the side of the tanks to maintain a digestate volume of 800 L. The float level switch was used to activate an electrical relay (American Zettler, Inc., Aliso Viejo, CA) routing power to a 1.27 cm full port solenoid-actuated 120-VAC PVC ball valve (Valworx, Inc., Cornelius,
NC) installed on 1.27 cm diameter PVC pipe placed 44 cm above the tank bottom that served as the waste outlet.

The top of each IBC tank was adapted to accommodate a 3-way luer valve and 6.35 mm tubing that served as a gas outlet and sampling port. The tubing was connected to a Wet Tip Flow Meter® (wettipgasmeter.com) by one arm of a 3-way luer valve fitting. The other arm of the fitting accommodated a syringe for taking samples for gas analysis. The side of the tank had an additional 0.635 cm diameter port with 2-way luer valve installed 34 cm above the tank bottom for taking liquid samples. All pipe and tubing connections to the tanks were made with Uniseal® pipe to tank fittings (US Plastic, Inc., Riverside, CA).

**Digester Operation**

Swine waste was obtained from a waste lagoon of a farrow to finish operation located in north-central Kentucky. Initially, 1,000 L of swine waste was pumped into each tank which activated the float switch controlled waste outlet as a means of partially concentrating wastewater solids and attaining the operating wastewater volume of 800 L. The experiments were conducted in duplicate as three separate trials of approximately 100 days each. In the first trial, the digesters were fed 290 g of a 2-parts ground corn to 1 part defatted soybean meal in 57 L of water twice weekly for a total of 8.41 kg, in the second trial the digesters were fed 565 g corn twice or three times weekly for a total of 23.84 kg, and in the third trial the digesters were fed 700 g corn meal, later increased to 1 kg of corn meal twice or three times weekly for a total of 62.5 kg. All corn feed was antibiotic free as attested by the vendor and confirmed by chemical analyses.
Sample Collection and Processing

Six different sets of samples were collected during three trials and were analyzed to measure total bacterial population (16S rRNA), tet gene copies, tetracycline antibiotic concentrations, and gas measurements. The sample types included samples of swine waste lagoon that were used for the anaerobic digestion (designated as initial), a bulk of feed sample from the swine farm (feed), corn mixed with water to activate the digesters (corn), samples obtained from the digesters as overflow during the addition of the corn feed (effluent), samples obtained from the central port consisted of the liquid above the sludge (digestate liquid), and finally digestate solid samples obtained from the bottom of the digesters at the end of each trial (sludge). Samples from the IBC tanks were collected at a port that fed into the center of the tank and at a dump connected to the top of the tank that was used to remove overflow.

Quantification of Tetracycline Resistance Genes

Total community DNA was extracted from 500 µl of the liquid samples (swine lagoon waste, effluent and digestate liquid samples) or 500 mg of the solid samples (swine feed, corn and sludge), using the FastDNA Spin kit for soils (MP Biomedical, Santa Ana, CA) following the manufacturer’s instructions. Real time quantitative PCR (qPCR) was used to quantify the concentrations of genes encoding for all bacteria (through 16S rRNA), and seven tet genes (tetA, tetB, tetG, tetM, tetO, tetQ, and tetW). The tet genes we targeted were commonly reported from swine feces and swine waste lagoons. The tetA, tetB, and tetG encode for efflux proteins while tetM, tetO, tetQ and tetW encode for ribosomal protection proteins. For the qPCR assays we used published primers, probes and protocols (Table 1). The primers were obtained from
Integrated DNA Technologies (IDT, Coralville, Iowa), and the dual-labeled black hole quencher probes for the 16S rRNA assay were from Biosearch Technologies, Inc. (Petaluma, CA). The qPCR assays and conditions were conducted according to the report by Cook et al. The assays were performed in Qiagen HotStarTaq Master Mix (Qiagen, Valencia, CA) for taqman probe assays QuantiTect SYBR green master mix (Qiagen, Valencia, CA) for SYBR assays in a total reaction volume of 25 µL. The assay consisted of 12.5 µL of the reaction mix, 1.5 µL of 10 µM (600 nm total primer concentration) each of the forward and reverse primers, 1 µL (100 nm total probe concentration) of probe and 1.5 µL of 25 mM MgCl2 (for 16S rRNA only), and 5 µL of 1:100 diluted sample DNA (diluted in 1:100 ratio to reduce the effect of PCR inhibitors in the samples) or the standard (ranging from 10¹ to 10⁸ copies), and 2 µL (for 16S rRNA only) or 4.5 µL (for SYBR assays) of water. Typical qPCR reaction consisted of initial activation at 95°C for 15 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing at specific temperatures (see Table 1) for 20 s, followed by final extension at 72°C for 30 s. Melt curve analysis was conducted between 65°C to 95°C with an increment of 0.2°C for 1 s. All qPCR reactions were run on the Bio-Rad CFX 96 real-time PCR detection system (Bio-Rad, Hercules, CA).
Table 1. Sequences, target sizes and melting temperature of primers used for quantitative real-time PCR for the quantification of tetracycline resistance genes (*tet*) under anaerobic digestion system.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer name</th>
<th>Primer sequence (5'-3')†</th>
<th>Tm (°C)‡</th>
<th>PCR product (bp)§</th>
<th>Assay type¶</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>16S-1055-F</td>
<td>ATG GCT GTC GTC AGT ACG GGC GGT GTG TAC</td>
<td>58.0</td>
<td>337</td>
<td>TaqMan</td>
<td>(Harms et al.²⁹)</td>
</tr>
<tr>
<td></td>
<td>16S-1392-R</td>
<td>FAM-CAA CGA GCG CAA CCC-BHQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16S-Probe-F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>tetA</em></td>
<td>tetA-F</td>
<td>GCT ACA TCC TGC TTG CCT TC CAT AGA TCG CCG TGA AGA GG</td>
<td>57.0</td>
<td>210</td>
<td>SYBR</td>
<td>(Ng et al.³⁰)</td>
</tr>
<tr>
<td></td>
<td>tetA-R</td>
<td></td>
<td></td>
<td></td>
<td>SYBR</td>
<td></td>
</tr>
<tr>
<td><em>tetB</em></td>
<td>tetB-F</td>
<td>CTC AGT ATT CCA AGC CTT TG GTA ATG GGC CAA TAA CAC CG</td>
<td>56.0</td>
<td>284</td>
<td>SYBR</td>
<td>(Sengelov et al.³¹)</td>
</tr>
<tr>
<td></td>
<td>tetB-R</td>
<td></td>
<td></td>
<td></td>
<td>SYBR</td>
<td>(Ng et al.³⁰)</td>
</tr>
<tr>
<td><em>tetG</em></td>
<td>tetG-F</td>
<td>CAG CTT TCG GAT TCT TAC GG CAA TGG TTG AGG CAG CTA CA</td>
<td>59.0</td>
<td>169</td>
<td>SYBR</td>
<td>(Ng et al.³⁰)</td>
</tr>
<tr>
<td></td>
<td>tetG-R</td>
<td></td>
<td></td>
<td></td>
<td>SYBR</td>
<td></td>
</tr>
<tr>
<td><em>tetM</em></td>
<td>tetM-F</td>
<td>GTG CCG CCA AAT CCT TCC TG GCA TCC GAA AAT CTG CTG GG</td>
<td>59.0</td>
<td>250</td>
<td>SYBR</td>
<td>(Szczepanowski, et al.³²)</td>
</tr>
<tr>
<td></td>
<td>tetM-R</td>
<td></td>
<td></td>
<td></td>
<td>SYBR</td>
<td>(Vikram et al.³³)</td>
</tr>
<tr>
<td><em>tetO</em></td>
<td>tetO-F</td>
<td>ACG GAR AGT TTA TTG TAT ACC TGG CGT ATC TAT AAT GTT GAC</td>
<td>58.0</td>
<td>170</td>
<td>SYBR</td>
<td>(Aminov et al.³⁴)</td>
</tr>
<tr>
<td></td>
<td>tetO-R</td>
<td></td>
<td></td>
<td></td>
<td>SYBR</td>
<td></td>
</tr>
<tr>
<td><em>tetQ</em></td>
<td>tetQ-F</td>
<td>AGA ATC TGC TGT TTG CCA GTG CCG AGT GTC AAT GAT ATT GCA</td>
<td>59.0</td>
<td>166</td>
<td>SYBR</td>
<td>(Aminov et al.³⁴)</td>
</tr>
<tr>
<td></td>
<td>tetQ-R</td>
<td></td>
<td></td>
<td></td>
<td>SYBR</td>
<td></td>
</tr>
<tr>
<td><em>tetW</em></td>
<td>tetW-F</td>
<td>GAG AGC CTG CTA TAT GCC AGC GGG CGT ATC CAC AAT GTT AAC</td>
<td>59.0</td>
<td>168</td>
<td>SYBR</td>
<td>(Aminov et al.³⁴)</td>
</tr>
<tr>
<td></td>
<td>tetW-R</td>
<td></td>
<td></td>
<td></td>
<td>SYBR</td>
<td></td>
</tr>
</tbody>
</table>

† Probe sequences each contained a 5' FAM fluorophore and 3' black hole quencher combination for use in probe-based 5' nuclease assays; probe concentration of 100nM; primer concentration of 600nM.
‡ Tm (°C) is the annealing temperature at which the PCR assay was performed.


§ PCR product refers to the expected amplification product size in nucleotide base pairs (bp).
¶ Refers to type of PCR assay used: TaqMan® or SYBR® green are quantitative, real-time PCR assays run on the Bio-Rad CFX 96 Real-time PCR detection system (Bio-Rad, Hercules, CA).

Analysis of Tetracycline Antibiotics

Chemicals

Tetracycline (≥98.0% purity), oxytetracycline hydrochloride (≥95.0% purity), chlortetetracycline hydrochloride (≥75.0% HPLC purity), 4-epitetracycline hydrochloride (≥95.0% purity) were all purchased from Sigma Aldrich (St. Louis, MO), demeclocycline hydrochloride (used as a recovery standard) was purchased from Alta Aesar (Haverhill, MA), while isochlortetraycycline hydrochloride was purchased from Toronto Research Chemicals (North York, ON, Canada). All solvents (methanol, acetonitrile, and water) were of HPLC or LC-MS grades, and were purchased from Sigma Aldrich. Sodium dihydrogen phosphate, ethylene diamine tetra-acetic acid disodium salt (Na$_2$-EDTA), citric acid monohydrate (all reagent grade) were purchased from Sigma Aldrich. Standards of all chemicals were individually dissolved in methanol and then mixed to prepare calibration curves ranging from 0.1 to 2 µg/mL.

Sample Extraction for Tetracycline Antibiotic Concentration Measurements

The samples for tetracycline antibiotics measurements were extracted from the anaerobic digester samples following a method developed by Capone et al. with minor modifications. Briefly, 10 mL of each sample was transferred into a 50-mL centrifuge tube, spiked with 100 µL of a 10 µg/mL standard of demeclocycline (DMC), followed by addition of 10 mL of methanol and 10 mL of a 0.2 M EDTA-McIlvaine buffer, pH 4.0 solution. The sample was vortexed for 30 s, sonicated for 15 min, and vortexed again for
30 s. Next, the sample was centrifuged at 4,500 rpm for 10 min and the supernatant was decanted into a 50-mL beaker. The pH of the supernatant was adjusted to pH 4.0 using concentrated sulfuric acid. Note that a few digester samples were less than 10 mL in volume, but for the sake of completeness, those samples were extracted by adjusting the volume of the solvent to be added.

The samples were then cleaned using weak cation exchange polymeric solid phase extraction (SPE) cartridges (Strata-X-CW, 500 mg, 6 mL, Supelco, Bellefonte, PA). The cartridges were first conditioned with 10 mL of methanol followed by 10 mL of water. After conditioning, the extracts were passed through the cartridges at a flow rate of 1 mL/min. The cartridges were washed with 20 mL of methanol: water solvent mixture followed by 10 mL of methanol, and then dried for 20 min. The tetracyclines were eluted with 6 mL (2 x 3 mL) of 0.1 M citric acid in 95% methanol. The eluents were evaporated to dryness at 50 °C under a gentle stream of nitrogen gas. The dry extracts were reconstituted in 1 mL of methanol, filtered with a 0.22 µm nylon filter, and transferred to 2 mL glass vials for LC-MS/MS analysis.

**LC-MS/MS Analysis**

Analysis of the sample extracts for the target tetracyclines was performed using a Varian 212-LC HPLC and Agilent 500 Ion Trap mass spectrometer detector (Agilent Technologies, Palo Alto, CA) with a Phenomenex C18 column (100 mm x 2.1 mm ID, 5 µm) at 45 °C; the injection volume was 20 µL. The mobile phase consisted of 0.1% formic acid in water (solvent A), and 0.1% formic acid in acetonitrile (solvent B). The mobile phase gradient started with a linear increase from 5% to 10% B for 1 min, followed by 10% B for 3 min, then ramped to 20% B for 5 min, then 20% B for 5 min,
and finally ramped back to 5% B for 1 min, for a total run time of 15 min. The flow rate was 0.25 mL/min. Flow was diverted from the MS for the first 3.5 min and last 1.5 min to minimize source contamination. Mass spectrometry data was collected in the positive ESI MS/MS mode. The source parameters were as follows: capillary voltage was set at 80 V, spray shield voltage at 600 V, needle voltage was at 5000 V, nebulizer temperature at 400 °C, nebulizer pressure at 40 psi, and drying gas flow rate at 1 mL/min. The parameters were optimized for the parent and daughter ions for each compound (Table 2). Note that the LC-MS could not detect all the 6 tetracyclines in a single run. Therefore, two injections were made for each sample. TC, ETC, OTC, and DMC were analyzed together, while CTC and ICTC were analyzed in a separate run.

Table 2. Optimized ion trap MS/MS parameters for the analysis of tetracyclines.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Parent ion (Da)</th>
<th>Daughter Ion (Da)</th>
<th>Collision (V)</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline</td>
<td>445</td>
<td>427</td>
<td>0.37</td>
<td>8.5</td>
</tr>
<tr>
<td>4-epitetracycline</td>
<td>445</td>
<td>427</td>
<td>0.37</td>
<td>6.9</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>461</td>
<td>443</td>
<td>0.44</td>
<td>7.5</td>
</tr>
<tr>
<td>Chlortetracycline</td>
<td>479</td>
<td>462</td>
<td>0.54</td>
<td>11.5</td>
</tr>
<tr>
<td>Isochlortetracycline</td>
<td>479</td>
<td>462</td>
<td>0.54</td>
<td>10.2</td>
</tr>
<tr>
<td>Demeclocycline (standard)</td>
<td>466</td>
<td>448</td>
<td>0.60</td>
<td>8.5</td>
</tr>
</tbody>
</table>

**Gas and Water Quality Measurements**

Gas production was measured daily during the workweek and averaged over the weekends. Gas and wastewater quality were measured weekly or biweekly as described in Loughrin et al. 86,87.

**Statistical Analysis**
Raw gene copy numbers were converted to concentrations per ml for liquid samples (port and dump samples and corn and water mix) or per gram of solid matter (sludge and swine feed). Concentrations were then transformed to $\log_{10}$ (number of gene copies/unit of sample + 1; hereafter referred to as gene concentration) to achieve normality of the data before statistical analysis. The $\log_{10}$ (number of gene copies/unit of sample + 1) transformation, instead of the commonly used $\log_{10}$ transformation, was necessary to account for the zero observations for some of the measured genes. Data from the two digesters were combined since the gene concentrations did not significantly ($P=0.1031$) differ between the two digesters. Mixed effects linear regression with the random effect of trial, and fixed effect of sample types was used to analyze the data. The mean concentrations of total bacteria and the tet genes in the swine feed, corn, dump, port and sludge were compared to their concentrations in the initial swine waste lagoon in order to evaluate the effect of anaerobic digestion. Pairwise comparisons of the marginal mean concentrations of the total bacteria and the tet genes were made between the sample types after adjusting for multiple comparisons by the Bonferroni method. All analyses were conducted in Stata/SE 15 (Stata Corp. College Station, TX).

RESULTS AND DISCUSSION

Tetracycline Resistance Genes

Mean concentrations of the genes encoding for total bacteria and tetracycline resistance are shown in Table 3. A single bulk feed sample, obtained from the swine farm that provided swine waste lagoon for the anaerobic digestion experiments, was analyzed for 16S rRNA and tet genes. The concentrations of the 16s rRNA, $tetM$, $tetQ$ and $tetW$ in the bulk swine feed sample were 9.41, 5.78, 4.70, and 4.33 respectively. The remaining
four tet genes (A, B, G and O) were not detected from the feed sample. The levels of total bacteria observed in the swine feed and samples of corn fed to the digesters were similar to that observed in the initial swine waste lagoon that was digested, and the samples collected during (effluent and digestate liquid) or after the digestion process (digestate or sludge). Similarly, swine feed and corn samples contained equal concentrations of tetM as the initial swine waste lagoon, effluent, digestate liquid and the sludge samples. Concentrations of tetQ and tetW in the bulk swine feed sample were similar to that observed in the corn, effluent and digestate liquid samples, but significantly lower than that of the initial swine waste and sludge. The corn samples contained similar concentration of tetA as the effluent and digestate liquid, but significantly lower than the initial swine waste lagoon or sludge samples. The concentration of tetB in the corn samples was similar to that of initial swine waste lagoon and sludge samples. The concentration of tetG in the corn samples was similar to the concentrations observed in the initial swine waste lagoon, effluent, digestate liquid and sludge samples. However, it contained significantly lower concentrations of tetO, tetQ and tetW as compared to that of the initial swine waste lagoon, effluent, digestate liquid or sludge samples.

Table 3. Concentration of gene copies for total bacteria (16S rRNA) and tetracycline resistance genes (tet) averaged across the sampling days and two bio-digesters adjusted for the random effect of three independent trials.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>16S rRNA</th>
<th>tetA</th>
<th>tetB</th>
<th>tetG</th>
<th>tetM</th>
<th>tetO</th>
<th>tetQ</th>
<th>tetW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swine feed</td>
<td>9.12AB</td>
<td>0D</td>
<td>0AB</td>
<td>0A</td>
<td>5.57A</td>
<td>0D</td>
<td>4.17AB</td>
<td>4.04AB</td>
</tr>
<tr>
<td>Corn</td>
<td>8.81AB</td>
<td>4.51A</td>
<td>5.71B</td>
<td>4.48ABC</td>
<td>5.17A</td>
<td>3.90D</td>
<td>3.71A</td>
<td>3.39A</td>
</tr>
<tr>
<td>Initial waste</td>
<td>9.19B</td>
<td>6.45C</td>
<td>5.39B</td>
<td>6.25C</td>
<td>5.84A</td>
<td>7.07B</td>
<td>8.18C</td>
<td>7.82E</td>
</tr>
<tr>
<td>Effluent</td>
<td>7.79A</td>
<td>4.70A</td>
<td>2.26A</td>
<td>3.77AB</td>
<td>5.84A</td>
<td>5.91A</td>
<td>6.79B</td>
<td>6.62BC</td>
</tr>
</tbody>
</table>
Concentrations of total bacteria and the *tet* genes did not significantly differ between the effluent and digestate liquid samples which were obtained during the digestion process. There was a 1.0 log$_{10}$ reduction of total bacteria in the effluent and digestate liquid samples compared to initial swine wastes which can be attributed to anaerobic digestion. We observed a 1.75 log$_{10}$, and a 1.23 log$_{10}$ reduction of *tet*A concentrations in the effluent and digestate liquid samples, respectively, from its initial concentration in the swine waste lagoon samples. Even more evident is the reduction of *tet*B by 3.1 log$_{10}$ in the effluent samples and by 2.3 log$_{10}$ in the digestate liquid samples, as compared to its concentration in the initial swine waste lagoon samples. The concentration of *tet*G was reduced by 2.5 log$_{10}$ in the effluent samples and by 2.0 log$_{10}$ in the digestate liquid samples, from its starting concentration of 6.2 log$_{10}$ in the swine waste lagoon. For *tet*O, statistically significant reduction by 1.2 log$_{10}$ was observed in the effluent, with no significant differences between the digestate liquid and initial swine waste lagoon. Concentration of *tet*Q was significantly reduced by 1.4 log$_{10}$ in the effluent and by 1.1 log$_{10}$ in the digestate liquid samples, from its concentration in the initial swine waste lagoon. The concentration of *tet*W was reduced by 1.2 log$_{10}$ in the effluent and by 0.83 log$_{10}$ in the digestate liquid samples, from its concentration in the initial swine waste lagoon. The sludge samples did not significantly differ from initial swine waste lagoon, effluent and digestate liquid samples with respect to the concentrations of total bacteria,

<table>
<thead>
<tr>
<th>Digestate-liquid</th>
<th>8.15$^A$</th>
<th>5.22$^{AB}$</th>
<th>3.12$^A$</th>
<th>4.25$^{AB}$</th>
<th>6.17$^A$</th>
<th>6.38$^{AB}$</th>
<th>7.13$^B$</th>
<th>6.99$^{CD}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sludge</td>
<td>8.63$^{AB}$</td>
<td>6.20$^{BC}$</td>
<td>3.34$^{AB}$</td>
<td>5.33$^{BC}$</td>
<td>6.86$^A$</td>
<td>7.04$^{AB}$</td>
<td>8.26$^C$</td>
<td>7.69$^{DE}$</td>
</tr>
</tbody>
</table>

Mean values with different letters in the column for each matrix-gene combinations are significantly different at the 5% level. Results are presented as mean values of the log$_{10}$ (gene copies/sample unit + 1).
tetA, tetB, tetG, tetM and tetO. Sludge samples had higher concentrations of tetQ and tetW compared to their corresponding concentrations in the effluent and digestate liquid samples, with no significant difference from that observed in the initial swine waste lagoons.

The mixed effects model analysis indicated the presence of significant variances among the trials for total bacteria and the tet genes. To see which gene-sample type combinations varied by trial, a univariate analysis including only trial as a factor was conducted for each gene by sample type. For the initial swine waste lagoon samples, the concentrations of six genes (16S rRNA, tetA, tetB, tetG, tetQ, and tetW) significantly (P<0.001) differed by trial with no difference for tetM (P=0.28) and tetO (P=0.25). For the corn samples, except for tetB (P=0.74) the quantities of the other genes were significantly (P<0.05) higher in the 2nd trial compared to the first trial (we note that corn samples were not analyzed in the 3rd trial). The measured genes did not differ among the three trials for the effluent samples (P>0.05). For the digestate liquid samples the quantities of tetA (P=0.03), tetB (P=0.008) and tetM (P=0.003) significantly differed by trial. For the sludge samples, only the concentrations of 16S rRNA (P=0.04) and tetG (P=0.03) marginally differed between trials; it was higher in trial 3 compared to trial 2 (we note that no sludge samples were processed in trial 1).

To examine the effect of sampling day we analyzed the data by trial (Table S1). In trial 1, for the corn samples the mean gene copies of 16S rRNA (P=0.69), tetA (P=0.59), tetB (P=0.86) and tetG (P=0.75) did not differ by day of application. However, the mean gene copies of tetM (P=0.03), tetO (P=0.01), tetQ (P=0.02) and tetW (P=0.02) significantly differed by date of application. Effluent samples were collected only on day
7; no sludge samples were collected and processed. The mean gene copies from the
digestate liquid samples did not significantly ($P > 0.05$) differ by sampling day. In trial 2,
the mean concentrations of the measured genes did not significantly ($P > 0.05$) differ over
time for all sample types. In trial 3, for the effluent samples sampling day had significant
effect on the concentrations of 16S rRNA ($P = 0.002$), $\text{tetA} (P = 0.03)$, and $\text{tetO} (P = 0.005)$;
there was no effect on the remaining $\text{tet}$ genes. Concentrations of all measured genes
from digestate liquid and sludge samples did not significantly ($P > 0.05$) differ by
sampling day.

In this study we investigated the effect of anaerobic digestion in reducing the
concentrations of total bacteria and tetracycline resistance genes in swine waste lagoon.
We evaluated its impact in two sample types (dump and port) collected during the
digestion process and at the end of the anaerobic digestion (sludge) in comparison with
the initial waste. We also measured concentrations of total bacteria and tetracycline
resistance genes in the swine feed obtained from the swine farm that provided the swine
waste lagoon, and samples of corn mixed with water and fed to the digesters to activate
the digestion process. We used qPCR to measure the concentrations of total bacteria and
tetracycline resistance genes from the total microbial community DNA extracted from the
samples. This culture independent metagenomics approach enables to quantify the total
ARGs (resistome) in a sample regardless of bacterial origin as opposed to culturing for
specific bacterial species.\textsuperscript{88,89} The culture independent approach overcomes the bias
associated with culture approach that can underestimate the effect of anaerobic digestion
since most bacteria in the anaerobic digesters cannot be cultured.\textsuperscript{88,90} One drawback of
the total community approach is that it does not differentiate whether the genes are from
live or dead bacteria. It can be possible that anaerobic digestion kills off bacteria, particularly aerobic bacteria, resulting in massive release of DNA into the external environment which leads to apparent increase in the concentration of genes when measured by qPCR. Even though anaerobic digestion could result in a shift in microbiota perhaps towards anaerobic bacteria, taxonomic profiling was beyond the scope of this project. However, the contribution of DNA from dead bacteria can be minimal in this study since the amount of average daily gas production as a result of bacterial activity reveals the integrity of the digesters. Furthermore, with respect to antibiotic resistance this may not be relevant since ARGs can be transferred horizontally even between unrelated bacterial species.

We did not observe any difference in the concentrations of total bacteria and tetracycline resistance genes between the dump and port samples implying that sample collection method during the digestion process does not have an effect in evaluating the impact of digestion in reducing total bacteria and ARGs. Dump samples were obtained from the valve connected to the top of the digesters as overflow that occurred during the addition of corn mixed in water to the digesters. The port samples were obtained by opening the valve attached to the bottom of the tanks. Anaerobic digestion resulted in statistically significant but biologically small (ranging from 1-2.5 log_{10}/ml) reduction in the concentrations of total bacteria and tetracycline resistance genes in the liquid (dump and port samples) components of the digested material from their initial concentrations in the original swine waste. Furthermore, anaerobic digestion did not result in significant reduction in the concentrations of total bacteria and tetracycline resistance genes in the solid component (the sludge) of the digested material. A lack of biologically meaningful
reduction in total bacterial biomass and ARGs, and the presence of detectable levels of
the tetracycline antibiotics can result in further dissemination of pathogens and antibiotic
resistant bacteria into the environment through the use of the digestates as soil
amendments, irrigation or when released into water stream\textsuperscript{90}.

**Concentration of Tetracycline Antibiotics**

Of the three target tetracycline antibiotics targeted, only TC and CTC were
detected in the swine digester waste samples analyzed, while OTC was not detected in
any sample. 4-Epitetracycline (ETC) and isochlortetracycline (ICTC), the main
metabolites for TC and CTC were detected in 62\% and 87\% of the total samples (n=57)
analyzed respectively. Further, ICTC was the most abundant (ranging from non-detect to
103 ppb) of all the target analytes for the 3 anaerobic digestion trials. The concentrations
of TC ranged from non-detect (ND)-36 ppb, ND-14 ppb, and ND-31 ppb for trials 1, 2,
and 3, respectively. The CTC concentrations ranged from ND-112 ppb and ND-103 ppb
for trials 1 and 3, respectively, while all the concentrations for CTC in all the trial 2
samples were below limit of detection. For the two metabolites studied, ICTC was the
most abundant, and its concentrations varied as follows: 0.37-11.9 ppm, 0.54-2.85 ppm,
and ND-103 ppb for trials 1, 2 and 3, respectively. The concentrations of ETC varied
between ND and 30 ppb for trial 1, ND and 7 ppb for trial 2, and ND and 19 ppb for trial
3.

There were no discernible trends in the concentrations of TC and CTC with
respect to time. Similarly, the same behavior was generally observed for the metabolites
ETC (trials 1, 2 and 3) and ICTC (trials 1 and 3). However, ICTC showed a different
trend in the trial 2 samples where its concentrations in both Tanks A and B initially increased with time and later decreased.

Recent research has focused on the metagenomics of ARGs in anaerobic systems and less on the fate of the antibiotics themselves. High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) often used in the more selective MS/MS mode, is the current ideal instrument for detecting low concentrations of antibiotics in difficult matrices such as swine wastes in anaerobic digester. HPLC-MS was used to determine TC, OTC, and CTC in swine manure treatment tanks in Japan. Although detection limits were not reported, measured sample concentrations ranged from 0.01 to 82.2ppm. The authors report the strong adsorption character of TCs to the solid sludge component of the manure.

The lack of discernible trends in the concentrations of TC and CTC with respect to time may be due to the fact that (a) a limited number of samples were extracted, and (b) many samples had concentrations below the detection limits. Similarly, the same behavior was generally observed for the metabolites ETC (in all trials) and ICTC (trials 1 and 3). However, ICTC showed a different trend in the trial 2 samples where its concentrations in both Tanks A and B initially increased with time and later decreased. This trend in ICTC concentrations is not surprising because it is a metabolite for CTC, therefore, its concentration should increase as CTC is degraded to ICTC. Subsequent degradation of ICTC will then lead to a decrease in the ICTC concentration. Arikan reported a 75% reduction in the concentration of CTC during 33 days of anaerobic digestion of manure from medicated calves. However, the concentration of its metabolite (isochlortetracycline) was increased by 100%. Arikan et al. found that the concentration
of OTC decreased by about 60%, while those of its metabolites (α-apo-oxytetracycline and β-apo-oxytetracycline) either decreased or increased during a 64-day period of anaerobic digestion of manure from medicated calves.

In a study investigating antibiotic (10 antibiotics including CTC, TC, and OTC) levels in swine and poultry waste and in water resources in proximal areas, swine waste storage lagoons typically contained antibiotic concentrations >100 µg/L. Antibiotics in nearby monitoring wells and streams were found in 31% and 67% of the samples taken near poultry and swine waste lagoons, respectively. Koike et al. also found ARGs originating from swine waste lagoons in the underlying groundwater wells.

**Relationship between Tetracycline Antibiotics and ARGs**

The presence of antibiotics such as tetracyclines even at sub-inhibitory concentrations in the anaerobic digesters can pose a selective pressure in the bacterial community. The maximum levels of tetracycline (16 ppb), and chlortetracycline (112 ppb) concentrations detected in the digester samples in this study are lower than the minimum inhibitory concentration of 16 µg/ml for tetracyclines according to Clinical Laboratory Standards breakpoint for tetracycline resistance. This means that even though these concentrations do not inhibit the bacterial growth, they are sufficient to exert selective pressure in the digester environment. Only the bacteria that acquire ARGs by way of plasmids will survive and multiply in those environments. Plasmids are a circular genetic structures independent of the bacterial genomic DNA that allows their host to adapt to harsh environments. Furthermore, one bacterial cell can contain one or thousands of these plasmids, often carrying ARGs, that are released into the environment upon cell death. Horizontal gene transfer (HGT) makes it possible to spread these
plasmids to other bacteria for survival. HGT is the transfer of genetic material between unicellular or multicellular organisms that is not transferred during reproduction, and through processes which include transformation, conjugation, and transduction. Horizontal gene transfer is evident by association of the ARGs with a mobile genetic element and insertion site co-location loss. Furthermore, animal waste management facilities such as bio-digesters promote the horizontal transfer of ARGs within the microbial community.

Previous studies reported the presence of ARGs and antibiotics in the environments impacted by animal manure. For instance, tetracycline resistance genes, and concentrations of tetracyclines ranging from 5.4 to 377.8 ppm were reported in soil samples near swine farms in three Chinese cities. The concentrations of tetracyclines in the soil samples reported by Wu et al. were much higher than the highest concentrations of tetracycline (36 ppb) and chlortetracycline (112 ppb) detected in the swine waste anaerobic digesters reported in our study. In a longer term study (6 years) of ARGs on manure treated farmlands in eastern China, nine classes of tetracycline resistant genes were detected in which some genes (tetB, tetW, tetC, and tetO) were reduced by composting, while others (tetG and tetL) were increased, however, the total abundance was relatively unchanged. In a study by Peak et al., tetracycline resistance genes (tetO, tetQ, tetW, tetM, tetB, and tetC) were measured for 6 months in cattle waste water lagoons. The authors reported that high-use lagoons that originated from cattle that received therapeutic, prophylactic or growth promoting levels of tetracyclines were found to have the highest levels of ARGs. Mixed-use lagoons, containing cattle waste from sick or quarantined animals contained the next highest levels of ARGs. This was followed by
no-use lagoons which were considered organic or quasi-organic in nature. This clearly indicates that concentrations of ARGs in the animal waste is correlated with the use of antibiotic in animal production. ARGs were found to fluctuate seasonally with a 10-100 times greater abundance in the autumn versus the winter. This seasonal effect can be associated with increased growth of bacteria during the warm seasons than the colder seasons a phenomenon which has been reported in beef cattle production.\textsuperscript{83} Furthermore, ARGs were found in two swine waste lagoons and the underlying ground water.\textsuperscript{101} The authors concluded that tetracycline resistance genes occur in the environment as a result of agricultural production which can therefore potentially enter the food chain.

We used a semi-batch (because the digesters were fed every few days) digester system in which swine waste perhaps with its associated ARGs, bacteria and antibiotics were added only as an initial seed at the beginning of each trial. This enabled us to evaluate the effect of a 100-day anaerobic digestion of the same waste material on ARGs. Under the continuous digester system, typical of commercial farms, animal wastes are continuously added to the digester which makes it practically impossible to evaluate the effects of interventions. We regularly added corn mixed in water into the digesters to promote the anaerobic digestion process. Total bacterial biomass and tetracycline resistance genes were also present in the swine feed and corn sometimes even at equal concentrations as the initial swine waste or the sludge. This indicates the widespread occurrence of bacteria and ARGs, and that animal feeds including corn and water can be important sources of resistant bacteria and their associated ARGs.\textsuperscript{102} Swine feed can be cross contaminated with antimicrobials from medicated feed at feed mills, during transport or at the farm.\textsuperscript{103}
Gas Production and Water Quality Measurements

The average daily total gas production, the concentrations of CO₂, CH₄, and chemical oxygen demand, and the pH in the anaerobic digesters greatly differed among the three trials (Table 4). Despite the great difference in the loading rates of the three trials, gas quality was similar with CO₂ averaging 21 percent of the mixture of CO₂ and CH₄. As feed loading rates increased, however, molar concentrations of both CO₂ and CH₄ increased. This was likely due to higher pressures in the tanks as daily gas production increased.

Table 4. Gas production and wastewater quality of three trials of anaerobic digesters.

<table>
<thead>
<tr>
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<th>Trial¹</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Gas Production</td>
<td></td>
</tr>
<tr>
<td>Average daily gas production (L)</td>
<td>33.1 ± 2.2</td>
</tr>
<tr>
<td>Carbon dioxide (µmole L⁻¹)</td>
<td>4,310 ± 325</td>
</tr>
<tr>
<td>Methane (µmole L⁻¹)</td>
<td>16,200 ± 1,220</td>
</tr>
<tr>
<td>Wastewater Quality</td>
<td></td>
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<tr>
<td>pH</td>
<td>6.16 ± 0.10</td>
</tr>
<tr>
<td>Chemical oxygen demand (mg L⁻¹)</td>
<td>1,180 ± 141</td>
</tr>
<tr>
<td>Bicarbonate buffering (µmole L⁻¹)</td>
<td>11.9 ± 1.4</td>
</tr>
</tbody>
</table>

¹ Data represent the mean ± standard error of the mean. Gas production measured daily and averaged over the weekend. Biogas and wastewater quality means represent the mean ± standard error of the mean of 32 determinations for trials 1 and 2, and 28 determinations for trial 3.

Daily gas production was greatly affected by loading rate, with trials two and three averaging approximately four and ten times that of trial one respectively. Chemical oxygen demand (COD) concentrations were only slightly higher in trial two than in trial one, however, only about three fold higher in trial three than in trial one. The pH
measurements in trials two and three were higher than in trial one and this was reflected in enhanced bicarbonate buffering.

As expected, gas production varied among the three trials as the feeding rate increased (Table 4). Thus, trial three produced approximately 10-fold more gas than did trial one while being fed approximately 7.5 times as much feed. Part of the increased gas production in trials two and three might be ascribed to more favorable environmental conditions in the digesters during trials two and three. The latter two trials had higher pH and bicarbonate buffering than did trial one, conditions that favor more stable digestion and higher gas production. Nevertheless, gas quality was good in all three trials, with methane averaging almost 80 percent. This was indicative of an active anaerobic digestion process.

In conclusion, 100 days of anaerobic digestion of swine waste lagoon did not result in a biologically meaningful reduction in the concentrations of tetracycline resistance genes. This was despite the fact that after adding the initial seed of swine waste containing ARGs, the digesters were fed antibiotic free corn meal. Similarly, tetracycline antibiotics and their metabolites persisted in the waste until the end of the digestion. The primary purpose of anaerobic digestion is the production of biogas for energy production with the secondary goal of waste volume reduction. Another highly desirable goal would be the reduction of ARGs in waste to reduce the spread of antibiotic resistant bacteria and associated resistance genes. Results from this study, however, indicate that anaerobic digestion is unlikely to reduce the spread of ARGs in the environment.

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ABBREVIATIONS USED

Antibiotic Resistance (AR)
Antibiotic Resistance Genes (ARGs)
Chemical Oxygen Demand (COD)
Chlortetracycline (CTC)
Demeclocycline (DMC)
4-Epitetracycline (ETC)
Ethylene diamine tetra-acetic acid disodium salt (Na₂-EDTA)
Food and Drug Administration (FDA)
Guidance for Industry (GFI)
High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS)
Horizontal Gene Transfer (HGT)
Intermediate Bulk Container (IBC)
Isochlortetracycline (ICTC)
LC-MS (Liquid Chromatography-Mass Spectrometry)
Major Facilitator Superfamily (MFS)
Non-detect (ND)
Oxytetracycline (OTC)
Real time quantitative PCR (qPCR)
Resistance Genes (RGs)
Solid phase extraction (SPE)
Tetracycline (TC)
Unites States Department of Agriculture (USDA)
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