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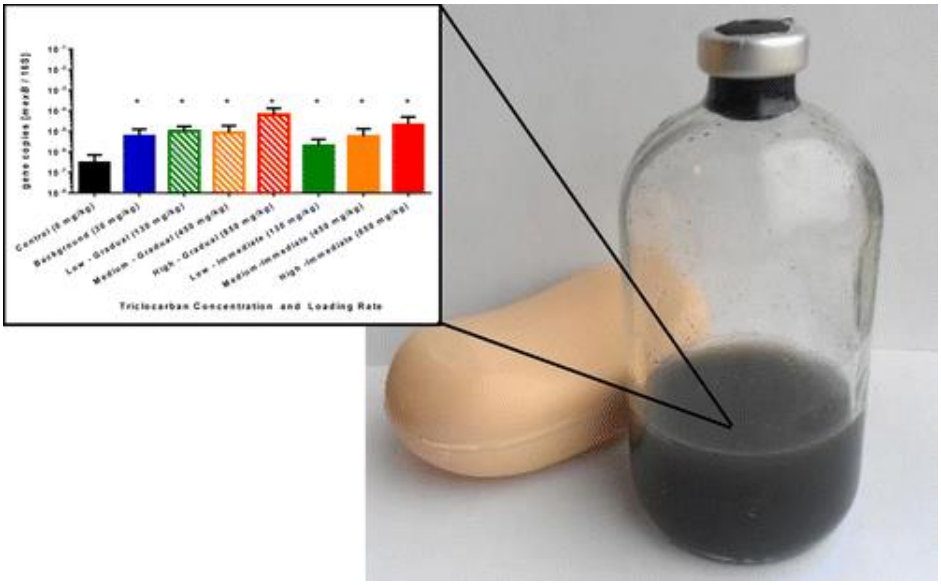
Abstract



Triclocarban (TCC) is one of the most abundant organic micropollutants detected in biosolids. Lab-scale anaerobic digesters were amended with TCC at concentrations ranging from the background concentration of seed biosolids (30 mg/kg) to toxic concentrations of 850 mg/kg to determine the effect on methane production, relative abundance of antibiotic resistance genes, and microbial community structure. Additionally, the TCC addition rate was varied to determine the impacts of acclimation time. At environmentally relevant TCC concentrations (max detect = 440 mg/kg), digesters maintained function. Digesters receiving 450 mg/kg of TCC maintained function under gradual TCC addition, but volatile fatty acid concentrations increased, pH decreased, and methane production ceased when immediately fed this concentration. The concentrations of the *mexB* gene (encoding for a multidrug efflux pump) were higher with all concentrations of TCC compared to a control, but higher TCC concentrations did not correlate with increased *mexB* abundance. The relative abundance of the gene *tet*(L) was greater in the digesters that no longer produced methane, and no effect on the relative abundance of the class 1 integron integrase encoding gene (*intI1*) was observed. Illumina sequencing revealed substantial community shifts in digesters that functionally failed from increased levels of TCC. More subtle, yet significant, community shifts were observed in digesters amended with TCC levels that did not inhibit function. This research demonstrates that TCC can select for a multidrug resistance encoding gene in mixed community anaerobic environments, and this selection occurs at concentrations (30 mg/kg) that can be found in full-scale anaerobic digesters (U.S. median concentration = 22 mg/kg, mean = 39 mg/kg).

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Introduction

Triclocarban (TCC) is a polychlorinated, binuclear, aromatic antimicrobial agent commonly used in bar soaps, detergents, cosmetics, and other personal care products to prevent products from cultivating bacteria and spoiling.(1, 2) Following consumer usage, TCC typically flows to wastewater treatment plants (WWTPs), and approximately 275 000 kg of TCC are sent to WWTPs each year.(3) TCC is not readily biodegraded in WWTPs, and approximately 75% of the TCC that enters a WWTP partitions to the biosolids.(4) In a U.S. nationwide survey on micropollutants in biosolids, TCC was found in 100% of municipal biosolids at a median concentration of 22 mg/kg and an average concentration of 39 mg/kg.(5) Of the personal care products, pharmaceuticals and other analytes screened in this survey, TCC was detected most frequently and at the highest concentrations. The high abundance of TCC is concerning because it has been found to be persistent, toxic, and potentially bioaccumulative in biological systems.(2)

Because TCC is designed to act against bacteria, the pervasiveness of TCC in biological engineered and environmental systems could impact microbial antibiotic resistance profiles.(6, 7) To date, very little research is available that describes the impacts of TCC on antibiotic resistance. Triclosan (TCS), which is also a polychlorinated, binuclear, aromatic antimicrobial agent, is a chemical analog of TCC and has been studied much more thoroughly for its impact on antibiotic resistance.(2, 8) Specific molecular targets of TCS in *Escherichia coli* were discovered in 1998, and since then, multiple TCS resistance mechanisms have been found in many bacterial genera.(9-12) The most prevalent forms of resistance to TCS are efflux through surface proteins, cell wall modification, and mutation of the target protein FabI, a key enzyme in the fatty acid elongation cycle.(8) Pathogenic and environmental bacteria have been shown to exhibit these resistance properties toward TCS, as previously reviewed.(8)

Of greatest concern is that resistance acquired by exposure to TCS or TCC could lead to cross-resistance to antibiotics.(13, 14) Indeed, the expression of an efflux pump which confers TCS resistance

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can lead to resistance to antibiotics with similar physicochemical properties.(13, 15, 16) Although little research has been performed to determine links between TCC and antibiotic resistance, many authors acknowledge TCC may present the same concerns as TCS when referring to cross-resistance to antibiotics.(7, 17) Son et al. found that TCC, as well as triclosan (TCS), selected for *tet*(Q) in aerobic activated sludge microcosms.(13) Resistance to TCC is most likely to occur in environments where TCC is pervasive at subinhibitory concentrations. Anaerobic digesters could be prime environments where TCC exerts selective pressure on antibiotic resistance genes (ARGs) because TCC sorbs to biosolids in a WWTP, and these biosolids are often stabilized using anaerobic digestion.(18) Moreover, retention times in anaerobic digesters are not long enough for significant biological transformation of TCC, yet the retention times are much longer than other unit operations providing bacteria a longer exposure time to adapt to TCC.(4, 19, 20)

The objective of this research was to determine if TCC impacts the abundance of ARGs as well as the microbial community structure and function in anaerobic digesters. Lab-scale anaerobic digesters were operated for 110 days with concentrations ranging from the background TCC levels found in the seed biosolids to inhibitory TCC concentrations that were twice the maximum concentration reported in the nationwide biosolids survey.(5) An additional goal of this research was to determine if the rate at which TCC concentrations increased in digesters would impact the ARG profiles and community structure. Various digester TCC concentrations were either immediately administered or attained after a gradual increase in TCC over approximately four solids retention times (SRTs). It was hypothesized that microbial communities that were provided more time to adapt to higher TCC levels would maintain function, have an increased abundance of ARGs, and exhibit community structure changes compared to communities that were immediately amended with increased levels of TCC.

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Materials and Methods

Experimental Setup

Lab-scale anaerobic digesters were operated for 110 days to determine the impacts of TCC loading rates and concentrations on ARGs and community structure. The digesters were inoculated with anaerobic digester biosolids taken from municipal digesters at South Shore Water Reclamation Facility (Oak Creek, WI). Background TCC levels were measured to be 27 ± 3 mg/kg (average ± average deviation of triplicate samples). Method details for TCC extraction and analysis by LC-MS are provided in the Supporting Information (section S1); recovery of ^{13}C-labeled TCC was 53% ± 10% (average ± standard deviation).

An SRT of 10 days was maintained. The digesters were 160 mL serum bottles with a 50 mL working volume and were capped with butyl stoppers. Each digester was fed daily with synthetic primary sludge at a loading rate of approximately 3.6 g COD/(L_r-day). The synthetic primary sludge consisted of dog food (Nutro Natural Choice, Franklin, TN) at 3% solids in a nutrient medium (Supporting Information, section S2). TCC was added to the synthetic primary sludge. TCC was dissolved in acetone then applied to a 1 cm layer of dog food and allowed to dry for 48 h. The dried dog food that contained the TCC was then mixed with the nutrient solution for daily digester feeding. Digesters were incubated at 35 °C and mixed on a shaker table at 100 rpm.

Eight sets of triplicate digesters were operated at different quasi steady-state TCC concentrations and ramp-rates (Figure 1) to test if stress induced by TCC would result in an increase in ARG abundance. Three different stress levels (in addition to the background level) were tested: a low level that did not inhibit digester function, a medium level that moderately inhibited digester function, and a high level that severely inhibited digester function. The medium and high concentrations were determined based on preliminary anaerobic toxicity tests and equated to the concentration which inhibits 10% of methane production (450 mg/kg) and concentration which inhibits 50% of methane production (850 mg/kg), respectively (see the

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Supporting Information, section S3, for descriptions of anaerobic toxicity tests and results). All digester sets were maintained with the background TCC concentration detected in the seed biosolids (30 mg/kg) for the first 45 days with the exception of the control digesters that received no TCC. After 45 days, five different quasi steady-state TCC concentrations were used and labeled as control (0 mg/kg), background (30 mg/kg), low (130 mg/kg), medium (450 mg/kg), and high (850 mg/kg). The low concentration was equivalent to the 95th percentile environmental concentration of TCC found in a nationwide survey of biosolids (i.e., 5% of samples surveyed were at concentrations of 130 mg/kg or higher).(5) The medium concentration in this study was nearly equivalent to the environmental maximum concentration detected in the EPA biosolids survey of 440 mg/kg, and the high concentration was approximately twice the environmental maximum concentration.(5)

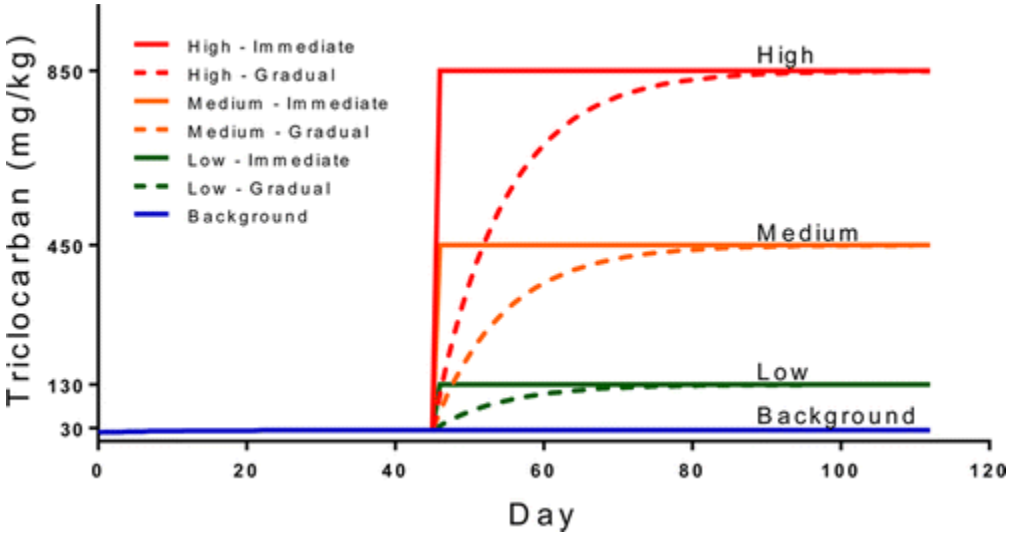

Figure 1. Nominal TCC concentrations (normalized to total solids) in triplicate sets of lab-scale anaerobic digesters. Not shown is a set of triplicate control digesters that received no TCC. All digesters, other than the control, were allowed to acclimatize to TCC feed at background concentrations that matched the TCC concentration of the original biosolids seed for 45 days before further addition of TCC.

On day 46, the contents of three sets of digesters were immediately amended with TCC to their nominal TCC quasi steady-state concentration, and TCC was continuously added to maintain this concentration for the duration of the experiment. These digester sets are referred to as low-immediate, medium-immediate, and high-immediate. Three other sets of digesters were fed TCC more gradually

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such that the nominal TCC concentration was not reached until after approximately three SRT values. These digester sets are referred to as low-gradual, medium-gradual, and high-gradual. Digester TCC concentrations were measured at days 0, 33, 47, and 110. All measured values were within 20% of expected concentrations. See the Supporting Information, section S1, for measured TCC values.

Molecular Methods

Microbial sampling and DNA Extraction

Approximately 1.8 mL of biomass slurry from each digester was taken prior to TCC addition on day 45 and on days 105, 107, and 110 after quasi steady-state conditions were established. DNA was extracted using MP FastDNA SPIN kits (Solon, Ohio) and modified to include three freeze–thaw cycles for improved lysis as described previously.(21) This extraction method may have an inherent bias toward the extraction of Bacterial DNA over Archaeal DNA.(22)

Detection and Quantification of ARGs

ARGs were quantified for differences between TCC and control digesters at quasi steady-state. Quantitative PCR (qPCR) was carried out on several genes. The gene *mexB* was selected because it is part of the MexAB-Opr multidrug efflux pump that has been associated with triclosan resistance;(21, 23) *tet*(L) was selected because it is also an efflux pump;(24) *erm*(F) was selected as a negative control because it is not an efflux pump, rather it confers macrolide resistance through methylation and was therefore not anticipated to be selected for by TCC;(25) the integrase of class 1 integrons (*intI1*) was selected as an indicator of horizontal gene transfer.(23, 26) These ARGs and *intI1* were normalized to the bacterial 16S rRNA gene.(27) Primers, annealing temperatures, efficiencies, limits of quantification, and qPCR conditions are given in the Supporting Information, section S4.

Illumina Sequencing and Bioinformatic analysis

Sequencing of partial 16S rRNA gene amplicons and analysis was done to evaluate the microbial community structure of digesters,

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and analysis was performed according to previously described protocols.(28, 29) Universal primers targeting the V4 variable region of 16S rRNA, 515F and 806R, were used for PCR amplification with HotStarTaq Plus Master Mix Kit (Qiagen). PCR conditions consisted of 94 °C for 3 min, followed by 28 cycles of 94 °C for 30 s, 53 °C for 40 s and 72 °C for 1 min, and a final elongation at 72 °C for 5 min. PCR product was purified utilizing Ampure XP beads. The purified PCR product was used to prepare DNA libraries by following the Illumina TruSeq DNA library preparation protocol. Sequencing was performed at MRDNA (Shallowater, TX) with Illumina MiSeq v3 300 base pair sequencing platform (Illumina, San Diego, CA). Raw unjoined sequence data were quality filtered (Q25). Barcodes and primers were removed from reads. Further sequences were removed including those with ambiguous base reads, those with fewer than 200 base pairs, and those with homopolymer sequences of 7 base pairs or longer. The denoised sequences were then clustered in operational taxonomic units which have 97% similarity. Each taxonomical unit was then compiled into taxonomic "counts" and classified using BLASTn against a highly curated database derived from GreenGenes, RDPII and NCBI. Sequencing was carried out on 48 samples (1 sample was taken from each of the triplicate digesters for 8 different TCC conditions on day 45 and day 110).

Analytical Methods

Gas production from each digester was measured daily with a 150 mL wetted glass syringe. Approximately every 10 days biogas methane content was measured by gas chromatography and thermal conductivity detection (7890A, Angilent Technologies, Santa Clara, CA) using a method described previously.(30) Volatile fatty acids (VFAs) were measured using a GC-FID as described previously (GC System 7890A, Angilent Technologies, Irving, TX). The pH was measured using a pH meter and probe (Orion 4 Star, Thermo, Waltham, MA).

Analysis and Statistics

Average, standard deviation, and average deviation values were calculated using Excel, whereas one-way ANOVA and *t* test calculations were performed using GraphPad Prism (V 6.04) for methane

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production and relative gene abundance. Custom R scripts were used to perform dual hierarchal clustering (utilizing R commands hclust of covariance, heatmap, and gplots library) and nonmetric multidimensional scaling (nMDS) of anaerobic community sequence data gathered from Illumina.(31)

Results and Discussion

Influence of TCC on Anaerobic Digestion Performance

During initial steady state before TCC addition (days 31–44), the average COD conversion to methane was 90 ± 17% (average ± standard deviation) for all reactors, while average biogas methane concentration was 68 ± 2.5%. All reactors maintained a pH near neutral; pH data can be seen in Supporting Information, section S5. Following healthy digester operation at background TCC levels, addition of TCC resulted in decreased methane production at 850 mg/kg, but TCC concentrations of 130 mg/kg and below did not impact methane production (Figure 2). The control, background, low-level, and medium-gradual feed digester sets produced 67 ± 8.5 mL of methane per day (corresponding to a COD conversion rate of 90 ± 16%) during quasi steady-state operation through day 110, and methane production was not statistically different between these reactors (ANOVA, $p = 0.06$). The medium-immediate, high-gradual, and high-immediate digester sets produced only 3.0 ± 1.0 mL of methane per day (corresponding to a COD conversion rate of 4 ± 1%) between days 80 and 110. The high-immediate digesters received 850 mg/kg of TCC on day 45, and decreased methane production was observed on day 46. The observed decrease in methane production might indicate that TCC directly inhibits methanogens but could also stem from the inhibition of bacteria that convert larger VFA's to acetate causing the digesters to sour. This high-TCC digester set had an immediate drop in biogas production and an associated rise in VFA concentration and drop in pH (see Supporting Information, section S6, for VFA data). In the high-gradual digesters, a greater than 10% difference in average methane production (relative to the control) occurred by day 55 when TCC was approximately 560 mg/kg. By day 60, when TCC was only at 680 mg/kg, average methane production had decreased by over 90% (t test, $p < 0.05$). Although the decrease

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in methane production was more gradual than observed in the high-immediate digesters, these digesters also had a rise in VFA concentrations and a drop in pH, and both digesters sets seemed to cease function due to a secondary buildup of VFAs. These conditions were too extreme for the microbial communities to successfully adapt and maintain methane production.

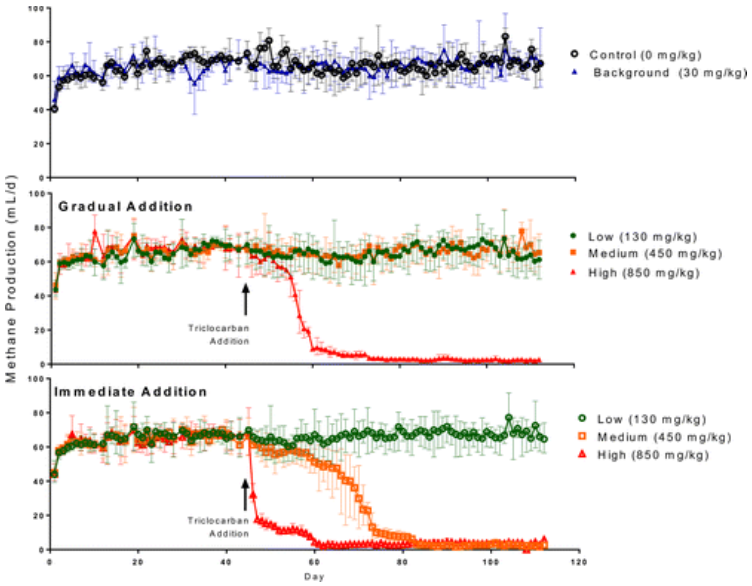

Figure 2. Methane production over 110 days of operation. Data points represent average values from triplicate digesters, and error bars represent range of data. Total biogas production can be seen in the Supporting Information, section S9.

For the medium TCC concentration of 450 mg/kg, the TCC loading rate determined whether methane production was maintained (Figure 2). In the medium-immediate digesters, methane production nearly ceased, while the medium-gradual digesters maintained methane production throughout the experiment. The microbial communities in the gradual digesters were able to adapt to the slow buildup of TCC from 30 to 450 mg/kg over three SRT values. The medium-immediate digesters were shocked with a 15 fold increase in TCC and did not have adequate time to adapt to this concentration of TCC; this digester set also had increased VFA concentrations (Supporting Information, section S6). An increase in VFAs is common following shock additions of toxicants.(32, 33)

On the basis of the functional data in Figure 2, full-scale anaerobic digesters should be able to maintain methane production if

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TCC concentrations increase slowly over time. The slow ramp-up used in this experimental study, as opposed to the immediate addition, is more similar to how concentrations would likely increase in full-scale anaerobic digesters if consumer usage and population density increases. These results imply that bacteria will have time to adapt to increasing TCC concentrations up to a certain threshold. This ability to acclimate to TCC is fortunate from a digester health standpoint because the medium concentration of 450 mg/kg used in this study that required acclimation time is similar to the environmental maximum concentration of 440 mg/kg already detected in biosolids. The slower ramp-up in TCC concentration to 850 mg/kg in the high digesters, however, did not allow the microbial communities to adapt and maintain function. In fact, the high-gradual digesters became inhibited well below the 850 mg/kg level as methane production was substantially reduced when TCC was only at 680 mg/kg. This inhibitory concentration is less than 2 times the environmental maximum detect, so it is feasible that full-scale digesters could see these levels if consumer usage continues. It is noted that the 50th percentile concentration of TCC estimated in biosolids is only 21.7 mg/kg, and the 90th percentile concentration is 88 mg/kg, which means the TCC concentrations in the majority of anaerobic digesters are still below inhibitory concentrations.(5)

Influence of TCC on Abundance of ARGs and intI1

The continued functioning of the lab-scale anaerobic digesters upon the addition of TCC might be explained by the proliferation of TCC resistance mechanisms through horizontal gene transfer within the microbial community or the selection of individual bacteria with established resistance to TCC. Resistance mechanisms have been identified for other biocides, such as TCS and quaternary ammonium compounds, and many of these mechanisms also produce resistance to antibiotics.(9, 34, 35) Efflux pumps are a resistance mechanism to many small molecules, including antibiotics, and the pumps are capable of eliciting cross-resistance.(14) For these reasons, the antibiotic resistance genes encoding efflux pumps, *mexB* and *tet*(L) found in bacteria, were investigated in this study.

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The relative abundance of *mexB* was statistically higher in all TCC digesters during quasi steady-state relative to the control, as seen in Figure 3 (ANOVA, $p < 0.05$); gene concentrations normalized to digester volume can be found in the Supporting Information, section S7. The abundance of *mexB* in high-gradual digesters was significantly higher than that in the background digester set as well ($p < 0.05$). TCC may have acted directly or indirectly to select for the presence of the *mexB* gene, but increases in TCC concentrations did not consistently correlate with an increase in the relative abundance of *mexB*. As these data are a measurement of *mexB* gene copies, it is possible that expression of *mexB* increased as TCC concentration increased; alternatively, *mexB* may be capable of providing resistance up to a threshold concentration of TCC, beyond which, other resistance mechanisms become dominant. Also noteworthy is that the presence of the *mexB* gene was not sufficient for the anaerobic digesters to maintain healthy function. This gene was likely maintained in bacteria that were not critical for digester function, and moreover, bacteria that had critical roles in maintaining function did not carry sufficient resistance mechanisms.

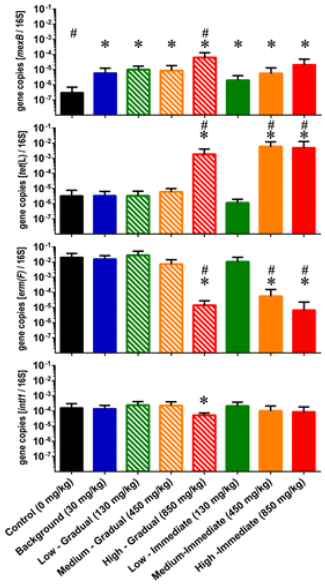

Figure 3. Abundance of ARGs and *intI1* at quasi steady-state. Each gene was normalized to 16S rRNA gene copies. Averages are shown with standard deviations of log values ($n = 9$, triplicate digesters were sampled on three different days during quasi steady-state: days 105, 107, and 110). An asterisk (*) denotes a statistical difference between the sample noted and the control ($p < 0.05$); (#) denotes a statistical difference between the sample noted and the background digester set,

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which maintained a TCC concentration equivalent to what was found in the seed biosolids throughout the experiment ($p < 0.05$).

This research demonstrates that TCC can select for a multidrug resistance gene in anaerobic environments, and this selection occurs at concentrations that were observed in full-scale anaerobic digesters. The *mexB* gene encodes for the MexB subunit of the MexAB multidrug efflux pump.(36, 37) The MexAB system is able to pump antibiotics, organic dyes, detergents, and organic solvents from within a cell and can decrease bacterial susceptibility to several classes of antibiotics and TCS.(37, 38) The genera *Pseudomonas* and *Cupriavidus*, along with other bacteria, are known to carry the *mexB* gene.(39, 40) Results from Zhang et al., suggest that *mexB* is found on plasmids as well and may be mobile in the environment.(41)

The presence of TCC in anaerobic digesters could be selecting for multidrug-resistant bacteria. The proliferation of the *mexB* gene has been observed in anaerobic digesters previously in response to biocides. In short-term 17-day experiments, the *mexB* gene was selected for in anaerobic digesters as a response to TCS at 500 mg/kg but not at 50 mg/kg.(21) In the longer-term experiments on TCC presented in this study, however, *mexB* selection occurred at background levels of 30 mg/kg. While the abundance of ARG's can be decreased during stabilization techniques such as lime stabilization or air drying beds, they still persist when biosolids are land-applied to the environment.(41, 42) No research is available to describe the impacts of biosolids stabilization specifically on the *mexB* gene.

The abundance of the *tet*(L) gene was substantially increased (Figure 3) under TCC loading conditions that also resulted in decreased pH (Supporting Information, section S5) and methane production (Figure 2). The relative abundance of *tet*(L) gene copies was at least 3 orders of magnitude higher in the inhibited digesters (high-gradual, high-immediate, medium-immediate) than in the control digesters ($p < 0.05$). The relative abundance of *tet*(L) was not statistically different in any of the uninhibited digesters (ANOVA, $p = 0.47$). The pH in the inhibited digesters dropped from approximately pH 7 to approximately pH 5 following high TCC additions (Supporting Information, section S5). Therefore, the low pH was likely the selective pressure that selected for genera carrying the *tet*(L) gene, and TCC did not

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specifically select for *tet*(L). The importance of conditions associated with a drop in pH, as opposed to solely the TCC levels, on the selection of *tet*(L) is supported by the results from the two medium digester sets that were receiving the same amount of TCC at quasi steady-state. The medium-immediate digesters, in which methane production nearly ceased because of the immediate addition of TCC, had an increase in relative abundance of *tet*(L) and lower pH. The medium-gradual digesters, which slowly received TCC, had no increase in *tet*(L), and neutral pH was maintained. Perhaps acid tolerant clades harbor *tet*(L) more frequently.

The *tet*(L) gene has not been previously implicated as a response to TCC or other biocides. In this study, because no difference existed between control digesters and TCC-amended digesters which maintained methane production, it can be concluded that TCC does not impact the abundance of *tet*(L) in digesters that maintain function. Previous research found mesophilic anaerobic digestion can actually decrease the abundance of *tet*(L) gene copies corroborating this result that healthy digester operation minimizes the discharge of *tet*(L) resistance genes.(24)

The *erm*(F) gene was quantified as a control because it encodes for macrolide resistance by altering the molecular target (23S protein) of erythromycin and was not expected to perpetuate from TCC exposure.(25) Indeed, the *erm*(F) gene was not enriched in the healthy TCC digesters relative to the control, but was selected against in digesters which were significantly inhibited. The decrease in *erm*(F) in the inhibited digesters was likely due to the shift in microbial community structure and function, similar to how *tet*(L) was increased in the inhibited digesters. Previous research found that functioning mesophilic anaerobic digesters did not influence the relative abundance of *erm*(F).(44)

One mechanism in which resistance gene abundance can be increased is through horizontal gene transfer mediated through class 1 integrons.(43, 45) The relative abundance of class 1 integrons was not different between any digester groups except for the high-immediate digester that was significantly different, albeit lower, than the control ($p < 0.05$). Based on the results found in Figure 3, TCC did not select

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for *intI1*. The similar relative abundance of *intI1* indicates equal potential in the digesters for bacteria to transfer genetic material through integrons.

The Impact of TCC on Microbial Community Structure of Anaerobic Digesters

The TCC concentrations and loading conditions that inhibited methane production also substantially altered the microbial community structure at the class and genus levels (Figure 4). Illumina sequencing generated an average of approximately 20 000 reads from each digester sampled. Significant differences in microbial community composition were observed in the inhibited digesters at the class level (Figure 4 bottom), while the digesters that maintained function were more similar. In the inhibited digesters, the Archaeal class Methanobacteria was enriched likely because the inhibited reactors had a pH of approximately 5.5, and some Methanobacteria are known to tolerate moderate acidity.(46) The Bacteria classes Actinobacteria and Clostridia were enriched in the inhibited reactors as well; both of these classes contain pathogenic bacterial strains which may have antibiotic resistance, and the Actinobacteria class contains many acid tolerant bacteria.(47, 48)

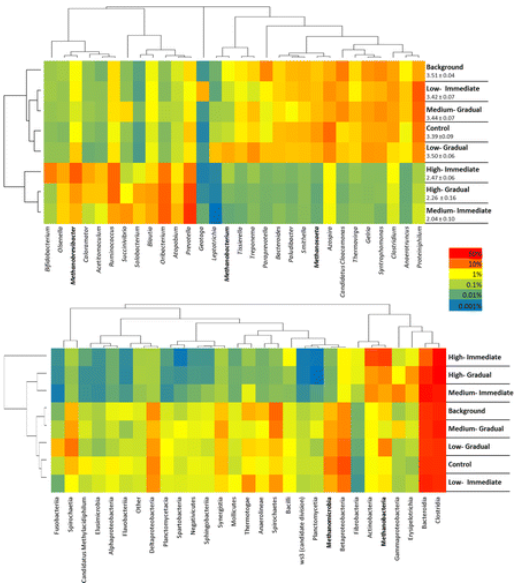

Figure 4. Dual hierarchal clustering of (bottom) averaged communities on day 110 (*n* = 3 for triplicate digesters) for class and (top) 30 most abundant genera. Coloring

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indicates the relative abundance of the class or genera within the digesters. Each group is evaluated using Krushkal–Wallis analysis of variance and cosine distances. Archaea are shown in bold. Shannon diversity index calculated using all genera for each sample is reported under each digester label (average ± average deviation).

In a dual hierarchal clustering of the 30 most abundant genera, digesters that continuously produced methane grouped together and were different than inhibited digesters in which methane production nearly ceased (Figure 4 top). The Shannon-diversity indexes were greater in all of the healthy digester sets compared to the digester sets where methane production decreased. Specifically, the genera *Prevotella* was highly selected for in the inhibited digesters. *Prevotella* are common members of the vaginal and ruminal microbiome and some species have been shown to display resistance to antibiotics.(49-51) *Prevotella* are found abundantly in digesters which include a preacidification step and were likely selected in this study because of their tolerance to low pH, and perhaps because of previously acquired resistance mechanisms.(52) In the uninhibited digesters, *Proteiniphilum* was detected at higher abundance. This genera encompasses acetate-producing organisms, which have been found in anaerobic digesters that treat protein rich brewery waste; it is suspected these organisms were enriched because the dog food feed was high in protein.(53)

Microbial community shifts may be responsible for adaptation to TCC and increased resistance in functioning digesters. An nMDS plot that includes all digester sets can be found in Supporting Information, section S8; the differences in community structure between the functioning and failing digesters is so stark that differences among the functioning digesters cannot be distinguished. The community structures of functioning digesters were further analyzed by nMDS (Figure 5). On day 45, when digesters had not yet received increased TCC loadings above background levels, the communities were very similar based on heavy overlap of 95% confidence ellipses (Figure 5a). By day 110, when communities had received TCC at different levels for over 6 SRT values, the communities diverged (Figure 5b). The control digesters were different than all digester sets at the 95th percent confidence interval except for the TCC background digester set. The low TCC digesters and the medium-gradual digesters all shifted away from the background digester set, and thus were distinctly different from the control and background digesters. These shifts in community

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structures suggest that the microbial communities shifted toward bacteria that were more resistant to TCC. In general, communities shifted away from the control as the TCC concentration increased.

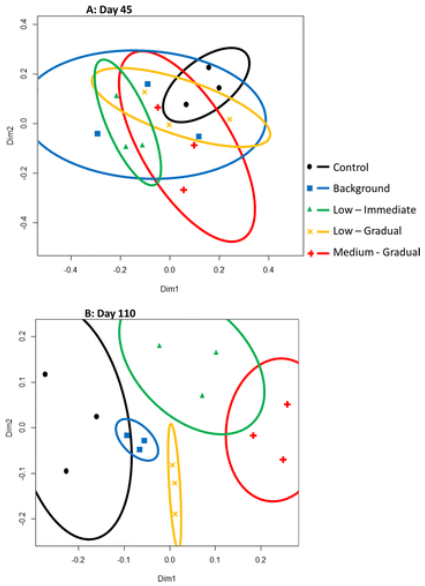

Figure 5. nMDS of all genera data generated for digesters that maintained methane production on (a) day 45 and (b) day 110. Ellipses represent 95% confidence intervals for the three points (each group represent the three triplicate digesters).

When considering the nMDS plot along with sequencing results, the shifts in the TCC-communities away from the control communities stemmed from changes in several genera. A Kruskal–Wallis test revealed that 52 genera had significant differences among these reactors ($p < 0.05$), but only 7 genera were represented by more than 1% of the population (47 genera made up a total of 1.1% of the average population of healthy reactors). The genera which represented more than 1% of the average healthy population were: *Candidatus cloacamonas* (5.7%,), *Proteiniphilum* (12.4%,), *Methanobacterium* (1.1%), *Paraprevotella* (3.0%), *Bacteroidales* (1.1%), *Azospira* (7.9%), and *Thermovirga* (2.2%). These genera may represent some of the major genera which TCC selects for (*Methanobacterium, Candidatus cloacamonas*) or against (*Bacteroidales, Azospira)*, and may contribute to TCC resistance in a digester. The healthy functioning TCC digesters also all had a greater fraction of *Bifidobacterium*, *Olsenella*, *Methanobrevibacter*, *Oribacterium*, *Atopobium*, *Ruminococcus*, and *Blautia* relative to the control. Conversely, the healthy functioning TCC digesters had lower fractions of *Clostridum*,

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Proteiniphilum, *Paludibacter, Smithella, Thermovirga,* and *Methanosaeta* relative to the control.

Implications

To better understand the impacts of TCC on public health and engineered systems, TCC needs to be further investigated for its role in impacting antibiotic resistance and microbial community structure, specifically in anaerobic digesters where TCC often resides. The results of this research suggest TCC is already present in anaerobic digesters at concentrations that act as a selective pressure for or against antibiotic resistance. The abundance of the multidrug efflux pump encoded by the *mexB* gene was at least an order of magnitude higher in all lab-scale anaerobic digesters that received TCC when compared to a control. The selection for *mexB* occurred at a TCC concentration (30 mg/kg) that is the same order of magnitude as the national median (22 mg/kg) and mean (39 mg/kg) concentrations.(5) This is the first research to show TCC can select for a multidrug resistance gene in a mixed anaerobic microbial community. Further research using metagenomics needs to be conducted to determine if *mexB* is the only ARG for which TCC enriches. Additionally, research should be conducted to determine if removing TCC as a stressor can reduce the abundance of the *mexB* gene to better understand how changes in consumer usage can alter ARG profiles in digesters. We are currently pursuing these questions using lab-scale anaerobic digesters.

In the lab-scale digesters where high concentrations of TCC resulted in high levels of VFAs, decreased pH, and decreased methane production; the ratio of *tet*(L) genes to 16S rRNA gene copies increased by 3 orders of magnitude. Concentrations of 680 mg/kg of TCC resulted in a 90% decrease in methane production under the gradual loading conditions used in this study; concentrations as high as 441 mg/kg were found in a nationwide biosolids survey.(5) A doubling of the environmental maximum TCC concentrations could cause digester failure; however, the concentration of TCC in the majority of anaerobic digesters are well below toxic concentrations. Important questions to answer are (1) In which environments (e.g., anaerobic digesters, soils, sediments) and how many environments is TCC selecting for antibiotic resistance? (2) Is this resistance

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reversible? (3) How is TCC altering the dynamics of microbial communities in full-scale digesters and other real-world environments? and (4) Do TCC, TCS, and antibiotics have synergistic effects on antibiotic resistance in anaerobic digesters?

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b03080.

- Quantifying triclocarban in biomass, nutrient media fed to anaerobic digesters, triclocarban anaerobic toxicity tests, primers and qPCR data, reactor pH, digester VFA concentrations, abundance of genes normalized to digester volume, total nMDS, and digester biogas production (PDF)

The authors declare no competing financial interest.

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