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Altered Antibiotic Tolerance in Anaerobic Digesters Acclimated to Triclosan Or Triclocarban

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Abstract: Bench-scale anaerobic digesters were amended to elevated steady-state concentrations of triclosan (850 mg/kg) and triclocarban (150 mg/kg) using a synthetic feed. After more than 9 solids retention time (SRT) values of acclimatization, biomass from each digester (and a control digester that received no antimicrobials) was used to assess the toxicity of three antibiotics. Methane production rate was measured as a surrogate for activity in microcosms that received doses of antibiotics ranging from no-antibiotic to inhibitory concentrations. Biomass amended with triclocarban was more sensitive to tetracycline compared to the control indicating synergistic

inhibitory effects between this antibiotic and triclocarban. In contrast, biomass amended with triclosan was able to tolerate statistically higher levels of ciprofloxacin indicating that triclosan can induce functional resistance to ciprofloxacin in an anaerobic digester community.

Keywords: Cross-resistance, Antimicrobial, Ciprofloxacin, Tetracycline, Chloramphenicol

Abbreviations:
- CDC, Center for Disease Control;
- TCS, triclosan;
- TCC, triclocarban;
- USEPA, United States Environmental Protection Agency;
- IC_{50}, concentration that inhibits 50% of methane production;
- COD, chemical oxygen demand;
- SRT, solid retention time;
- ATA, anaerobic toxicity assay;
- pATA, prokaryotic anaerobic toxicity assay;
- K_{ow}, octanol water partition coefficient;
- VFA, volatile fatty acid

1. Introduction

Antibiotic resistance is a growing public health concern resulting in thousands of deaths every year (CDC, 2013). Antibiotic resistance is influenced and stimulated by many types of stressors including antibiotics, antimicrobials, and metals in a variety of environments (Alanis, 2005, McNamara et al., 2014 and Carey and McNamara, 2015). Of particular concern is the development of cross-resistance whereby resistance to one stressor results in resistance to another stressor (Sefton, 2002). Calls to prudently prescribe antibiotics and minimize their use in agriculture stem in part from desire to quell promotion of cross-resistance and the corresponding spread of 'superbugs'.
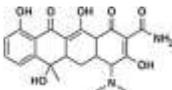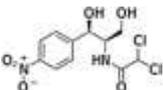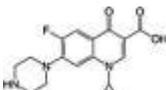
Beyond prescription antibiotics, resistance to household antimicrobials has been documented, which yields concern that cross-resistance to clinically relevant antibiotics could develop from antimicrobial-Bacteria interaction. TCS and TCC are two household antimicrobial chemicals found in a range consumer products including antibacterial soaps. Cross-resistance to antibiotics stimulated by TCS has been investigated and discovered in many pathogenic bacteria (Giuliano and Rybak, 2015 and Saleh et al., 2011). Although less investigated for its impact on cross-resistance to pathogens, similar concerns for TCC arise (Carey et al., 2016a and Chalew and Halden,

2009). The majority of cross-resistance studies have been conducted on pure-cultures, and research documenting cross-resistance in mixed communities is lacking.

The high concentrations of TCC and TCS in municipal wastewater and anaerobic digesters pose a particular concern because of their interaction with a rich and diverse community of Bacteria. TCC and TCS were the most abundant pharmaceuticals (mean concentration of 39,433 µg/kg and 16,097 µg/kg respectively) found in US wastewater biosolids out of 145 chemicals surveyed (USEPA, 2009). Digesters contain a sub inhibitory mixture of antimicrobials and antibiotics that renders development of cross-resistance a strong possibility. Resistance development in biosolids is suspected to have a critical role in antibiotic resistance in terms of public health (Munir et al., 2011, Pruden, 2013 and Ju et al., 2016).

The objective of this research was to determine if long-term TCC or TCS exposure in anaerobic digesters impacted functional resistance (as measured by methane production) to antibiotics. Specifically, the toxicity level (IC_{50}) of tetracycline, ciprofloxacin, and chloramphenicol was measured in anaerobic microcosms to determine if exposure to these antimicrobials made microbial communities more or less susceptible to antibiotics. These antibiotics were chosen because they are all separate classes of antibiotics, have variable water chemistries, and are associated with cross-resistance to TCS (see Table 1). Furthermore, most cross-resistance mechanisms that have been identified are efflux pumps that have been upregulated (See Table 1), although it is possible horizontally transferred genes could be responsible for cross resistance.

Table 1. Antibiotic properties.

	Tetracycline	**Chloramphenicol**	**Ciprofloxacin**
Structure			
Class	Polyketide	Other[a]	Fluoroquinolone
Log K_{ow}	−1.37	1.14	0.28
Water solubility	231 mg/L @ 25 °C	2500 mg/L @ 25 °C	30,000 mg/L @20 °C
pKa	3.3	5.5	6.1

	Tetracycline	Chloramphenicol	Ciprofloxacin
Concentration in biosolids[b]	1914 µg/kg	NA[c]	6858 µg/kg
TCS link to cross-resistance	Cross-resistance to tetracycline forms from TCS exposure in pathogens (eg. *E. Coli*, *P. aeruginosa*)	Mechanisms associated with TCS resistance are also associated with ciprofloxacin resistance	Chloramphenicol resistance is stimulated by exposure to TCS in pathogenic bacteria (eg. *S. Maltophilia*, *S. enterica serovar Typhimurium*)
Genes associated with resistance to TCS and antibiotic	acrAB (efflux), smeDEF (efflux), nfxB (efflux)	acrAB (efflux)	acrAB (efflux) nfxB (efflux)
References	Braoudaki and Hilton, 2004, Chuanchuen et al., 2001, Kappell et al., 2015, Sanchez et al., 2005 and Karatzas et al., 2007	Piddock, 2006	Birosová and Mikulásová, 2009, Braoudaki and Hilton, 2004, Karatzas et al., 2007, Sanchez et al., 2005 and Chuanchuen et al., 2001

[a]Chloramphenicol inhibits bacteria uniquely, but is somewhat related to macrolides.
[b]Mean concentration found in 72 treatment plants by McClellan and Halden, 2010.
[c]Chloramphenicol was not analyzed by McClellan and Halden, 2010.

2. Materials and methods

2.1. Acclimatizing mother reactors to TCC or TCS

Three mother digesters were established as a biomass source for testing antibiotic toxicity against antimicrobial-acclimatized anaerobic biomass: a control digester, a TCS-amended digester, and a TCC-amended digester. These digesters are referred to as 'mother digesters' throughout this manuscript because the biomass from these digesters was used for inoculum for the experiments that tested antibiotic toxicity. Biomass from these digesters was used to determine the concentration of antibiotics required to inhibit 50% of methane production during batch methanogenic assays with each amended biomass. Each mother digester had 4 L of working volume and was seeded with biomass from a full-scale mesophilic anaerobic digester at South Shore Wastewater reclamation facility (Oak Creek, WI). Biomass from this facility was previously measured to have TCC and TCS concentrations of approximately 30 mg/kg for both antimicrobials in March of 2014 (Carey et al., 2016a and Carey et al.,

2016b). The solid retention time of the mother digesters was 15 days and each digester was given 6 g of ground and sieved (40 mesh) dog food daily (1.8 g COD/L-d) in nutrient medium to simulate primary sludge. TCC and TCS was added to each feed to achieve steady state concentrations of 150 mg TCC/kg solids and 850 mg TCS/kg solids in the method outlined previously (control had no TCC or TCS addition) (Carey et al., 2016a). Digesters were operated for a total of 210 days. Qausi steady-state operation was established over the first 140 days (>9 SRT values), and biomass was used for toxicity testing over the remaining 70 days.

2.2. Prokaryotic anaerobic toxicity assay (pATA)

Anaerobic toxicity assay (ATA) style tests were performed to test the toxicity of three antibiotics (Stuckey et al., 1980). Methane production is measured as a surrogate for activity at different doses of a toxicant during batch tests. The experiments performed here differ from traditional ATAs in that a more complex feed carbon source was utilized (dog food) instead of acetate. Dog food was used because degradation to produce methane flows through all trophic groups (Bacterial and Archaeal) in an anaerobic digester. Although Archaea are widely thought to be immune to the actions of the inhibitors used in this experiment (i.e., antibiotics), chloramphenicol has been shown to inhibit Archaea (Hilpert et al., 1981), and the potential for minor or major inhibition in these experiments remains a possibility. Given that trophic groups from Bacteria or Archaea were potentially inhibited (as opposed to only Archaea in a traditional ATA), the modified assays that are performed in this work are referred to as "prokaryotic anaerobic toxicity assays" (pATAs), as prokaryotic refers to Bacteria and Archaea.

Prior to performing a pATA, waste biomass was collected from the mother digesters over a five day period. The biomass was allowed to degas for an additional 3 days before testing. For a given pATA test, a constant volume of biomass (50 mL) and a constant COD load (3.5 g COD/L) was employed for each glass serum bottle (160 mL) reactor. For these experiments, seven antibiotic doses were used in triplicate to span several orders of magnitude. The antibiotic employed ranged from no antibiotic to inhibitory concentrations that were determined

based on preliminary testing. Properties and associations of these antibiotics can be found in Table 1. Antibiotics were added directly to dog food in a solvent and allowed to dry before use in the pATA. Antibiotics were purchased from Sigma Aldrich (St. Louis, MO).

After each bottle was loaded with biomass, dog food, and antibiotic, the head space was purged with a 70/30 ratio of N_2/CO_2 gas and capped with an airtight butyl-rubber stopper. Biogas volume was measured every 6–24 h for approximately 20 days. When approximately 100 mL of biogas was produced in any given bottle, the methane percentage was determined with a gas chromatogram coupled to a flame ionization detector (Carey et al., 2016a). Methane production rate was then determined over a period of approximately 20 days. Results were interpreted by determining the linear methane production rate for each antibiotic dose. The concentrations of antibiotic that reduced methane production rate by 50% (IC_{50}) were determined with Prism® (GraphPad Software, La Jolla, CA). A higher IC_{50} value indicates a more resistant biomass to a given antibiotic.

3. Results and discussion

3.1. Mother reactor operation

All three mother digesters (control, TCC-amended, and TCS-amended digesters) maintained function, a pH of approximately 7–7.5 and VFA levels less than 60 mg/L (see Fig. 1).
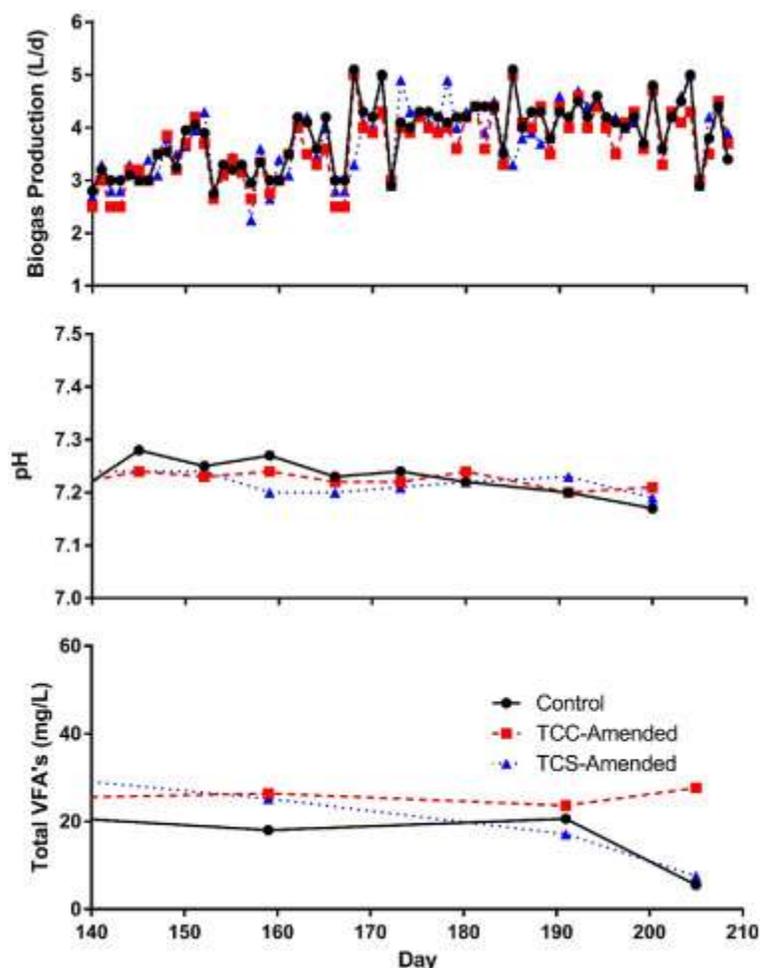
Fig. 1. Biogas, pH and total VFA concentration from day 140–210. Total VFAs is the sum of acetic, propionic, butyric, iso-butyric, valeric, and iso-valeric acid.

Biogas production was similar among all digesters, with average biogas production of 3.6 ± 0.6 L/day. Methane concentration in biogas was 68 ± 3.8% in control digesters, 66 ± 4.4% in TCC digesters, and 64 ± 5.0% in TCS digesters. Solids concentration in the digesters was at 9.5 ± 0.1 g/L after day 100 and was constant for all pATA tests. In total, three pATA tests were performed with initial biomass draws occurring on day 146 (tetracycline), 177 (chloramphenicol), and 199 (ciprofloxacin).

3.2. Tetracycline pATA

The TCC-amended biomass was more susceptible to inhibition by tetracycline relative to the control biomass; the IC_{50} value (Fig. 2)

was statistically lower than the control in test sets (control IC_{50} = 5700 mg/kg, TCC IC_{50} = 780 mg/kg). Some antibiotics have been shown to have synergistic inhibition effects on anaerobic digestion (Aydin et al., 2015 and Ozbayram et al., 2015). For example, tetracycline has greater inhibition of methanogenesis when used in combination with sulfamethoxazole or erythromycin (Aydin et al., 2015). The mother digester amended with TCC may have been operated at a threshold concentration of TCC, i.e., when this biomass was introduced to another chemical stressor (tetracycline in this case), the biomass was more readily inhibited. Tetracycline is known to work by inhibiting protein synthesis. TCC is thought to intercalate within the cellular membrane; it is possible that TCC made cell membranes more porous and allowed tetracycline to enter into cells more easily. It is difficult to parse the mechanism because there is a paucity of research regarding the mechanism of TCC inhibition on bacteria. Perhaps TCC selected for organisms that maintained the function of the anaerobic digester, yet were intrinsically more sensitive to tetracycline. Tetracycline impacted the TCS-amended biomass differently than the TCC-amended biomass. For TCS-amended biomass, no statistical difference was observed from the control. This result suggests that TCS and tetracycline inhibit cells by independent manners and the chemicals do not have synergistic inhibitory nor additive cross-resistance effects.
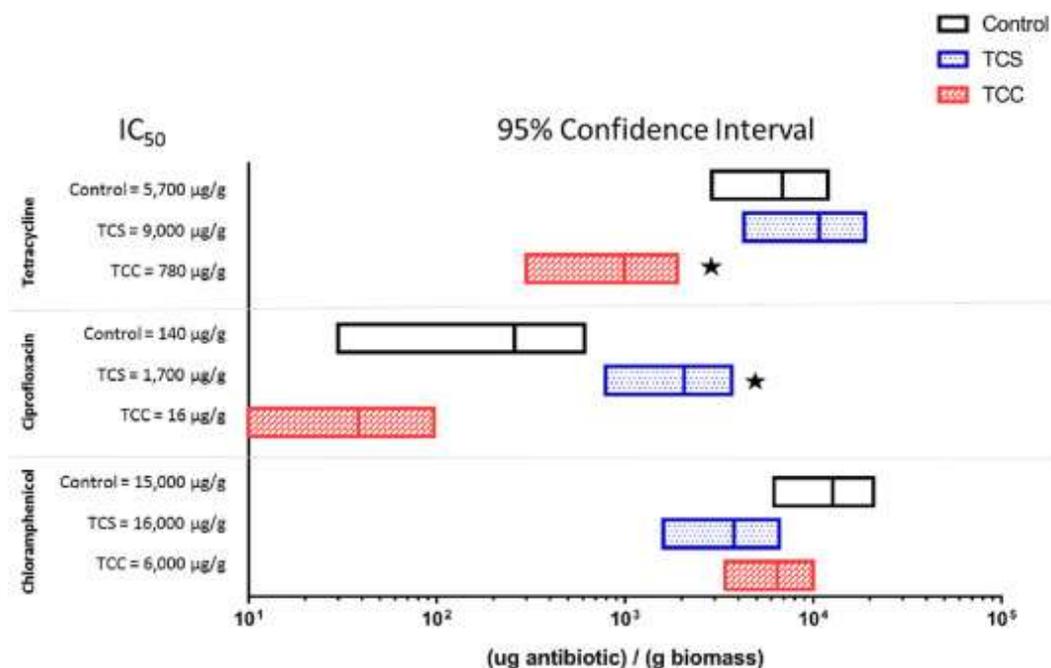
Fig. 2. Previous exposure to antimicrobials alters biomass tolerance to antibiotics; the bars represent the mean IC_{50} value with flanking 95% confidence intervals. The mean is also specified on the left side of the graph. Stars to the right of the bar indicate a statistically significant difference from the control, i.e. $p < 0.05$. A treatment group that is lower than the control indicates that the biomass became more sensitive to antibiotics after exposure to antimicrobials (such as TCC/tetracycline). A treatment group that is higher than the control bar indicates that the biomass became more resistant to antibiotics after exposure to antimicrobials (such as TCS/ciprofloxacin).

3.3. Ciprofloxacin pATA

TCS-amended digesters gained cross-resistance to ciprofloxacin as indicated by digesters having a statistically higher IC_{50} value than the control digesters. This result suggests that resistance mechanisms that allow bacteria to tolerate TCS also allow bacteria to tolerate higher concentrations of ciprofloxacin. In contrast, the IC_{50} of the TCC biomass was lower as observed with TCC and tetracycline; however, the 95% confidence intervals heavily overlapped, and this result was not statistically different. Resistance to fluoroquinolones (the family of antibiotics to which ciprofloxacin belongs) is well documented (Kern et al., 2000). While many of the resistance mechanisms rely on target mutation, efflux is also a known resistance mechanism against fluoroquinolones (Jacoby, 2005). In fact, some of the exact same efflux resistance mechanisms that resist ciprofloxacin have been found to resist TCS in pure culture experiments (McMurry et al., 1998).

Previous experiments showed that *Salmonella enterica* (a pathogenic bacterium) exposed to 0.5 mg/L of TCS had increased resistance to ciprofloxacin, and it was concluded that an efflux system (AcrAB) was responsible for this cross-resistance (Birosová and Mikulásová, 2009). Alternatively, TCS could have shifted the microbial community such that the digester still maintained overall function, but community members were intrinsically more tolerant to ciprofloxacin. Either selection of resistant bacteria or horizontal transfer of resistance genes (e.g. efflux pumps capable of expelling ciprofloxacin and TCS) stimulated by TCS could result in an overall increase in resistance to ciprofloxacin. This finding is especially important when considering that ciprofloxacin and triclosan were two of top three most abundant micropollutants detected in biosolids in a US survey (out of 72 analytes measured) (McClellan and Halden, 2010).

3.4. Chloramphenicol pATA

For chloramphenicol, the pATA did not yield statistically significant differences for TCC-amended or TCS-amended biomass compared to the control. The IC_{50} for the control biomass is higher than the TCS-amended and TCC-amended biomass (by at least 5000 µg/g); however, the 95% confidence intervals overlap heavily. The AcrAB efflux pump has been previously associated with both TCS and chloramphenicol resistance,but functional cross-resistance did not develop in these experiments (Piddock, 2006).

4. Conclusions

TCC increased the toxicity of tetracycline to anaerobic biomass while TCS induced functional resistance to ciprofloxacin in pATA tests. These results indicate that the mechanisms of action for TCC and TCS are different, however, both scenarios are of concern. For TCC, synergistic action with antibiotics is harsher on digester communities which could lead to a loss of function; for TCS, cross-resistance to antibiotics has public health implications since TCS is so widely used in households and disseminated throughout the environment.

Perhaps resistance may emerge to a class of antibiotics more quickly if cross-resistance is abundant in the environment, and if so,

this class of antibiotics should be given special attention in medical use, research, and risk assessment. Determining which classes of antibiotics are the most susceptible to gaining cross-resistance to the most abundant chemical stressors can help guide further research for the protection of public health.

Acknowledgements

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