Supplemental Information

Chronic exposure to triclosan sustains microbial community shifts and alters antibiotic resistance gene levels in anaerobic digesters

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S1. Nutrient Media

Table S1- Nutrient Media

Constituent	**(mg/L)**
NH_4Cl	400
$MgSO_4.7H_2O$	195
KCl	400
$CaCl_2.2H_2O$	50
$(NH_4)_2HPO_4$	80
$FeCl_2.4H_2O$	*40
$CoCl_2.6H_2O$	*10
KI	10
$(NaPO_3)_6$	10
$NiCl_2.6H_2O$	1
$ZnCl_2$	1
$MnCl_2.4H_2O$	0.5
NH_4VO_3	0.5
$CuCl_2.2H_2O$	0.5
$AlCl_3.6H_2O$	0.5
$NaMoO_4.2H_2O$	0.5
H_3BO_3	0.5
$NaWO_4.2H_2O$	0.5
Na_2SeO_3	0.5
$NaHCO_3$	6000
$Na_2S.9H_20$	300
L-Cysteine	10
*Yeast Extract	*10

*differences from Speece *et al.*[1]

Dog food was added at 30 g/L to nutrient media in order to deliver 3.6 gCOD/L-day

S2. Anaerobic inhibition testing of triclosan

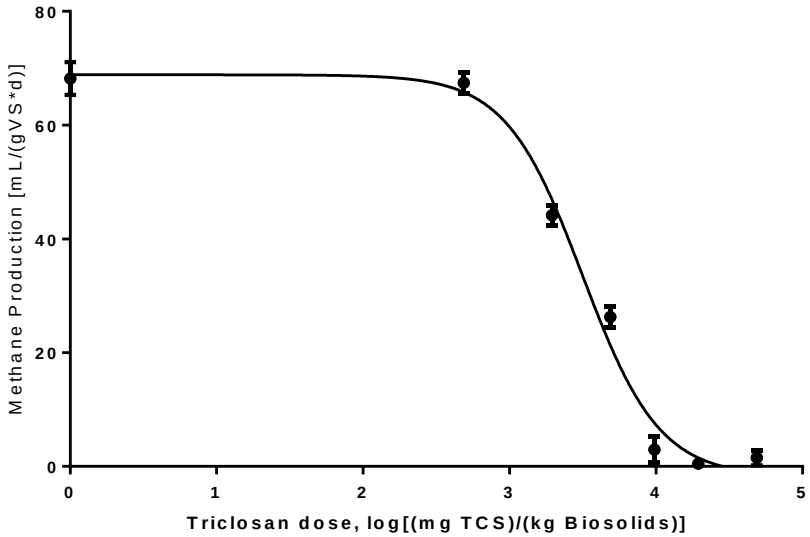

Figure S2- Steady state methane production at various TCS concentrations (n=3).

Inhibition testing for TCS was carried out using an anaerobic toxicity assay style test. Seven triclosan doses (0, 500, 2000, 5000, 10000, 20000, and 50000 [mg TCS]/[kg total solids]) were delivered to anaerobic digesters and methane production rate was measured. Briefly, triplicate anaerobic digesters were prepared for each triclosan dose with a 50 mL working volume in a 160 mL serum vial. Each digester initially received 3.8 g/L_r of calcium propionate. The headspace was sparged with a 70/30 mix of N_2/CO_2 and sealed with a pressure containing rubber butyl stopper. TCS was then added to reactors in 50 µL of Dimethyl Sulfoxide. Biogas production was measured by displacement with a wetted gas syringe. Methane fraction in the biogas was measured after 10 days by gas chromatography (7890A, Agilent Technologies, Irving, TX, USA), when headspace gas was assumed to be equal to biogas produced by the biomass.

S3. Triclosan concentration measured by LC/MS

Table S3- Recoveries and concentrations of TCS in Biosolids

Sample	**13-C TCS Recovery** (%)	**Nominal TCS concentration** (mg/kg)	**Measured TCS concentration accounting for recovery** (mg/kg)
Seed	57%	N/A*	28
Control (Day 45)	78%	0	0.96
Background (Day 45)	64%	30	17
Control (Day 110)	87%	0	0
Background (Day 110)	73%	30	15
Low (Day 110)	60%	100	74
Medium (Day 110)	68%	850	770
High (Day 110)	73%	2500	2990

* Measured in the seed biomass, therefore no concentration is expected

Five mL samples were collected of waste biomass when TCS was quantified. The sample was placed in a crucible and allowed to dry for 72 hours at 35°C; total solids concentration was determined from mass measurements.

The dried biomass was scraped from the crucible, and a known mass was extracted using Accelerated Solvent Extraction System (Dionex 42 ASE, Thermo Scientific, Sunnyvale, CA, USA). Prior to extraction, the extraction cells were cleaned with a triple rinse of methanol, sonication in acetone, followed by another triple rinse with methanol (adapted from Anger *et al.*)[2]. The dried biomass was placed into the extraction cell and 2 µg of ^{13}C-labed TCS (Cambridge Isotope Laboratories, Inc, Andover, MA, USA) was added in a nonane solution and allowed to dry. The samples were then extracted by heating the cells to 60^{o}C while holding the pressure at 1500 psi using methanol as the solvent. The cells were heated through the cycle twice and 60% of the cell volume was collected after each cycle. The final extract volume was approximately 20 mL for each sample.

Liquid chromatography-mass spectrometry (LC/MS) was employed to measure the concentration of TCS and ^{13}C-TCS in biosolid extracts. Injection volumes of 20 µL were used on a Shimadzu LCMS-2020 (Shimadzu, Addison, IL, USA). A C18 column (Phenomenex Luna, 3 µm particle size, 150mm x 3mm) was used to perform chromatography. The mobile phase shifted linearly over a 13 minute runtime from 80/20 ratio of methanol/water to 100% methanol. The flow rate of the mobile phase was 400 µL/min. The M/Z ratios for detection on the mass spectrometer were 287 and 299 for TCS and ^{13}C-TCS, respectively. Peak interactions were accounted for when determining concentrations. TCS was assumed to be recovered at the same rate as ^{13}C-TCS and this recovery was applied in the calculation for TCS concentration in biosolids.

S4. qPCR and primer information

Table S4- qPCR conditions and efficiencies

Gene	Annealing Temperature	Forward Primer & Reverse Primer	Efficiency Average	Limit of Quantification (copies/µL)	Ref.
16S	60°C	For. (5'-CCTACG GGAGGCAGCAG-3') Rev. (5'-ATTACCGCGGCTGCTGG-3')	101.5%	10,000	[3]
mexB	63°C	For. (5'-GTGTTCGGCTCGCAGTACTC-3') Rev. (5'-AACCGTCGGGATTGACCTTG-3')	103.0%	500	[4]
intI1	60°C	For. (5'-CCTCCCGCACGATGATC-3') Rev. (5'-TCCACGCATCGTCAGGC-3')	94.9%	500	[5]
tet(L)	60°C	For. (5'-TCGTTAGCGTGCTGTCATTC-3') Rev. (5'-GTATCCCACCAATGTAGCCG-3')	88.2%	500	[6]
erm(F)	60°C	For. (5'-CAACCAAAGCTGTGTCGTTT-3') Rev. (5'-TCGTTTTACGGGTCAGCACTT-3')	86.6%	500	[7]

Quantitative polymerase chain reaction was carried out on a BioRad CFX Connect Real Time System (Hercules, CA). Each assay was performed with the following conditions: 95°C of initial denaturation for 10 min, 35 cycles of denaturation (30 s, 95°C) and combined annealing/extension (30 2, temperature specific to primer). The reaction volume was 20 µL containing the following: 10 µL of BioRad iTaq SYBR Green Supermix (Life Science Research, Hercules, CA), 5 µL water with optimized quantities of forward and reverse primers (1 nM for resistance genes and intI1 and 2 nM for 16S rRNA gene), and), 5 µL of diluted DNA (approximately 50 ng for resistance genes and 0.25 ng for 16SrRNA by mass).

Linear range standard curves were established for each gene. Positive standards for PCR were previously generated[8,9]. All samples were diluted by at least 50 to remove inhibitor substances and to fall within the range of standards. Data used within the study were used if the R^2 value was ≥ 0.95. Resistance genes and intI1 were below detection limits for the feed in all cases.

S5. Illumina processing information

Sequencing of the V4 region of the 16S rRNA gene was performed on community extracted DNA to evaluate the microbial structure of the digesters[10,11]. Preparation, sequencing, and bioinformatics were performed at MRDNA (Shallowater, TX, USA) with Illumina MiSeq v3 300 base pair sequencing platform (Illumina, San Diego, CA, USA). The 515F and 806R universal primer set were used with HotStarTaq Plus Master Mix Kit (Qiagen, USA) to perform PCR amplification prior to sequencing. The conditions used included 94°C denaturing for 3 minutes, for by a 3-step PCR (28 cycles) which consisted of 94°C for 30 seconds, 53°C for 30 seconds, and 72°C for 1 minute. A final elongation was performed at 72°C for 5 minutes. The PCR products were purified (Ampure XP beads), and then used to prepare libraries by following the Illumina TruSeq preparation protocol. A Q25 filter was applied to un-joined sequence data. Sequences were removed which had ambiguous base reads, less than 200 base pairs, and those with homopolymer sequences greater than 6. Operational taxonomic units (OTU) were clustered by sequences which have 97% or greater similarity. Each OUT was then classified using BLASTn against a database which is derived from GreenGenes, RDPII and NCBI. This analysis was carried out on 30 samples (triplicate of each reactor condition at day 45 and 110)

S6. NMDS of all communities

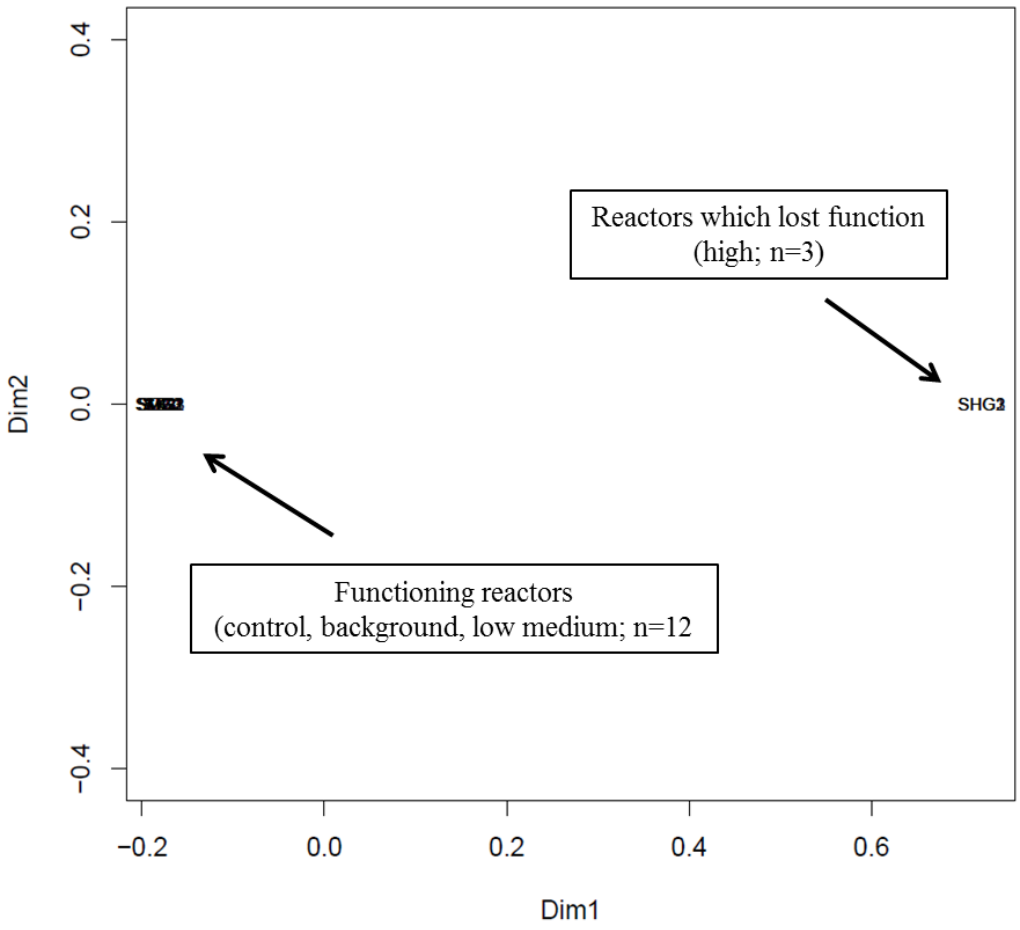

Figure S6- NMDS of day 110 communities performed with genus level data including reactors which lost function. Resolution of functioning reactors is not appropriate to make conclusions.

S7: 16S gene copies by volume

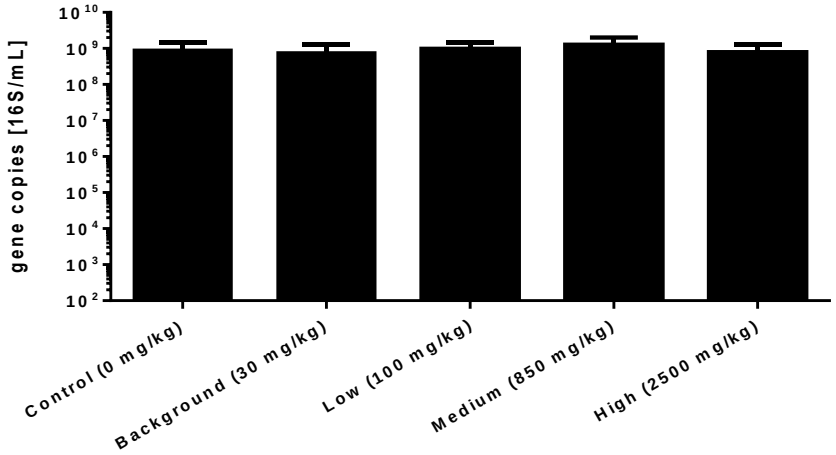

Figure S7- 16S copies per mL of digester. ANOVA testing indicates that the concentrations are not statistically different (p= 0.46, n=9). Extraction efficiency was not quantified.

Table S7: 16S rRNA gene copies detected in each sample normalized to volume (copies/mL)

Control (0 mg/kg)	Background (30 mg/kg)	Low (100 mg/kg)	Medium (850 mg/kg)	High (2500 mg/kg)
1.13E+09	1.06E+09	1.54E+09	1.83E+09	1.47E+09
1.53E+09	6.28E+08	1.42E+09	2.06E+09	1.38E+09
1.50E+09	9.05E+08	1.29E+09	1.34E+09	9.56E+08
4.95E+07	1.36E+08	1.06E+08	1.36E+08	1.23E+08
2.42E+08	9.16E+07	1.87E+08	1.18E+08	2.71E+08
1.84E+08	1.46E+08	2.78E+08	3.19E+08	1.67E+08
1.10E+09	1.64E+09	1.31E+09	1.76E+09	9.84E+08
8.61E+08	8.08E+08	1.22E+09	1.53E+09	5.66E+08
1.15E+09	1.21E+09	1.05E+09	1.83E+09	8.73E+08

S8: Operational Taxonomical Unit (OTU) data

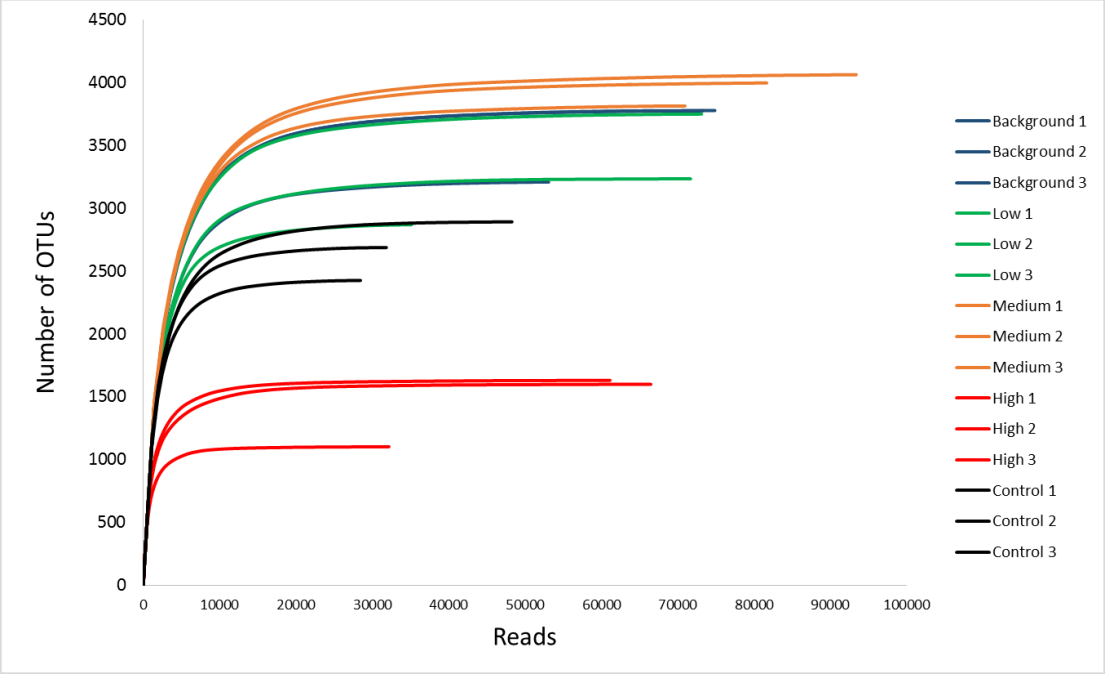

Figure S8.1- Rarefaction curve of OTU level data.

Table S8- Richness and diversity indicies

	Shannon Index[a]	Simpsons Index[b]	Chao1[c]	Total OTUs
Control 1	3.89	0.991	4433	2689
Control 2	3.48	0.991	3907	2427
Control 3	5.36	0.988	4533	2893
Background 1	5.99	0.992	4801	3210
Background 2	7.82	0.991	5453	3777
Background 3	7.92	0.990	5498	3780
Low 1	7.02	0.982	4766	3236
Low 2	4.31	0.994	4360	2871
Low 3	7.82	0.991	5566	3751
Medium 1	9.75	0.994	5938	4065
Medium 2	8.79	0.994	5557	3999
Medium 3	7.74	0.993	5571	3816
High 1	2.89	0.959	1735	1103
High 2	5.41	0.966	2469	1600
High 3	5.00	0.964	2618	1631

[a] A higher Shannon index represents more diversity

[b] A higher Simpson index represents less diversity

[c] A high Chao1 richness estimator indicates higher richness

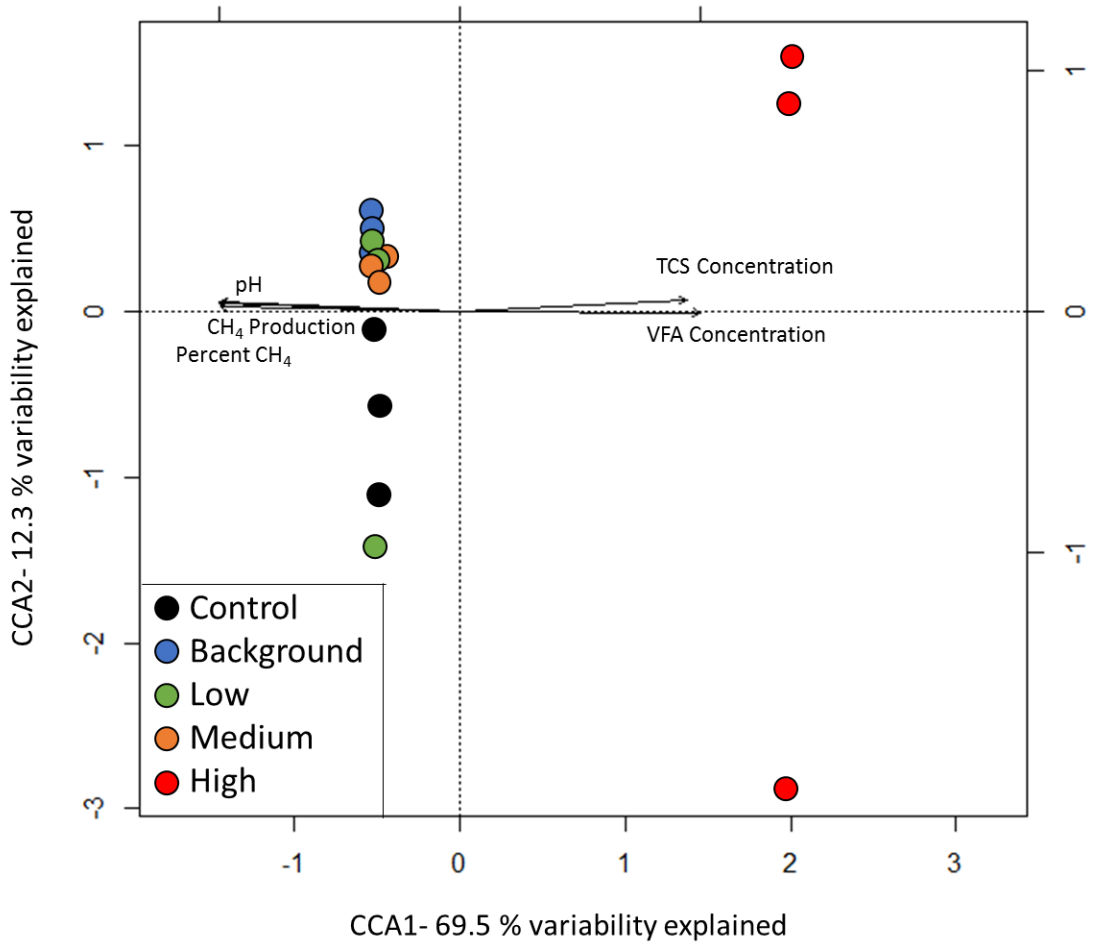

Figure S8.2- Canonical Correspondence Analysis (CCA) of OTU data from digesters constrained by pH, CH_4 Production, Percent CH_4, TCS Concentration, and VFA Concentration. Clear separation can be observed from the "High" digesters along the x-axis. Increased TCS concentration and increased VFA concentration were correlated with the High digesters. The high digester set had the highest TCS concentration (2500 mg/kg), VFA concentrations higher than 20,000 mg/L, a pH below 5, methane production of less than 5 mL/day, and less than 25% methane in the biogas. Conversely, increased pH, CH_4 Production, and Percent CH_4 correlated with the Control, Background, Low, and Medium digesters. These digester sets had VFA concentrations < 50 mg/L, a pH near 7, biogas was near 70% methane, and methane production was near 70 mL/ day. The horizontal axis can explain 69.5% of the variability in the data and all of the continuous variables used to constrain the data set correlate along this axis. Further, TCS concentration and increased VFA concentration were anti-correlated to increased pH, CH_4 Production, and Percent CH_4 which was expected.

S9. References

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