JOURNAL OF ENVIRONMENTAL SCIENCES 56 (2017) 164–168

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JES JOURNAL OF ENVIRONMENTAL SCIENCES www.jesc.ac.cn

Structure and activity of thermophilic methanogenic microbial communities exposed to quaternary ammonium sanitizer

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ARTICLE INFO

Article history:
Received 5 June 2016
Revised 31 August 2016
Accepted 8 October 2016
Available online 27 October 2016

Keywords:
Anaerobic digestion
Antimicrobials
Biofuels
Microbial ecology
Waste management

ABSTRACT

Food processing facilities often use antimicrobial quaternary ammonium compound (QAC) sanitizers to maintain cleanliness. These QACs can end up in wastewaters used as feedstock for anaerobic digestion. The aim of this study was to measure the effect of QAC contamination on biogas production and structure of microbial communities in thermophilic digester sludge. Methane production and biogas quality data were analyzed in batch anaerobic digesters containing QAC at 0, 15, 50, 100 and 150 mg/L. Increasing sanitizer concentration in the bioreactors negatively impacted methane production rate and biogas quality. Microbial community composition data was obtained through 16S rRNA gene sequencing from the QAC-contaminated sludges. Sequencing data showed no significant restructuring of the bacterial communities. However, significant restructuring was observed within the archaeal communities as QAC concentration increased. Further studies to confirm these effects on a larger scale and with a longer retention time are necessary.

Introduction

Anaerobic digestion is a waste management technology that can biologically convert wastewater organic matter to renewable gaseous biofuel, or biogas (Khalid et al., 2011; McCarty et al., 2011). Bacteria in digester sludge are responsible for the hydrolysis, acidogenesis and acetogenesis of complex organic compounds to acetic acid, carbon dioxide and hydrogen gas. These products serve as the substrates for the methanogenic archaea that ultimately convert them to methane and carbon dioxide, the principal components of biogas.

Quaternary ammonium compound (QAC) sanitizers are frequently used in food facilities due to their efficacy as a disinfectant (Gerba, 2015). Previous studies have shown that QAC contamination can affect methane production in mesophilic digesters operating at 35°C (Tezel et al., 2006, 2007). However, thermophilic digesters employing temperatures of 50–60°C are often used as well, which can have considerable differences in sludge microbial community structure (Chachkhiani et al., 2004; Shi et al., 2013) that can lead to more rapid cellulose degradation (Shi et al., 2013) and elevated

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http://dx.doi.org/10.1016/j.jes.2016.10.005
1001-0742/

methane production rates (Hashimoto, 1983). However, these benefits may come at the cost of decreased community stability (Dinsdale et al., 1996; Kim et al., 2002). To date, the susceptibility of thermophilic sludges to QACs has not been determined nor has the phylogenetic composition of QAC-contaminated sludge microbial communities been linked to biogas production data. In this study, the structure of thermophilic sludge microbial communities exposed to varying levels of QACs was determined via 16S rRNA gene sequencing. Changes in community composition were related to biogas production rate and quality data to identify microorganisms that may be sensitive to QACs.

1. Experimental

1.1. Anaerobic digestion

Batch anaerobic digesters were comprised of 250-mL glass media bottles fitted with modified caps containing a port connected to tubing and an in-line check valve (catalog #80103, Qosina). The methanogenic sludge used for these digesters was obtained from a thermophilic anaerobic digester located near the University of California, Davis campus that processes food scraps, spoiled packaged food, manure, yard waste, and paper waste. The digester did not process any rinse water from facilities using QAC sanitizers and thus the background QAC level in the sludge was assumed to be negligible. The sludge had a total solids content of 3.5%. The commercial QAC sanitizer F-29 (Rochester Midland Corporation, Rochester, NY, USA) was added to sludge at varying levels. The QAC content of F-29 sanitizer consisted of 4% (W/W) alkyl (C12–16) dimethylbenzylammonium chloride, 3% decyldimethyloctylammonium chloride, 1.5% didecyldimethylammonium chloride, and 1.5% dioctyldimethylammonium chloride.

To establish methanogenic cultures, sludge was initially incubated for 2 days at 55°C to exhaust most residual methane production. Each digester was then loaded with 100 mL of sludge and 0.5 mg of finely-milled tomato pomace to simulate organic matter that may be found in food processing wastewater. Varying volumes of F-29 sanitizer were loaded into digesters to achieve 0, 15, 50, 100 or 150 mg QAC/L. Reactor headspace was flushed with nitrogen gas. Reactors were incubated at 55°C for 4.5 to 7.25 days to elucidate differences in methane production between treatments without the confounding ecological effects of substrate exhaustion. Methane, carbon dioxide, and hydrogen content in biogas was measured via a MicroOxymax respirometry system (Columbus Instruments). The pH of the sludge was measured for two reactors from each treatment at the end of the incubation.

1.2. Deoxyribonucleic acid isolation and 16S rRNA gene sequencing

Genomic DNA was purified from sludge microbial communities using a PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc). The V4 region of the 16S rRNA gene was amplified and sequenced according to previously described methods (Simmons et al., 2014) with one alteration. Under the altered protocol, a qPCR library quantification kit (KAPA Biosystems) was used to determine the concentration of V4 amplicons capable of being sequenced ahead of sequencing.

1.3. Data processing and analysis

DNA sequencing reads were filtered, assembled, clustered, and assigned taxonomy using iTagger, a custom PERL script developed by the Joint Genome Institute, as described elsewhere (Hausmann et al., 2016). Ecological analyses were performed using RStudio (version 0.98.1103) with the vegan and entropart packages. Prior to analysis, singletons were removed from operating taxonomic unit (OTU) read count data to reduce noise. Linear regression analyses of community diversity, dissimilarity, OTU abundance, and biogas production data were performed using JMP software (version 12.0.1, SAS). For comparison of bacterial OTU changes in response to QAC concentration, critical P-values were adjusted for multiple comparisons to achieve a familywise error rate of 0.05 using the Bonferroni method (Bland and Altman, 1995).

2. Results and discussion

2.1. Biomethane production

A significant negative trend was observed between QAC level and methane production over the culture period (Fig. 1a, $p = 0.002$). Differences in cumulative methane production between treatments related to changes in methane production rates (Fig. 1b). Sludges containing 0, 15, or 50 mg QAC/L appeared to maintain more similar methane generation rates compared to sludges with 100 or 150 mg QAC/L. Specifically, sludges with at least 100 mg QAC/L showed a marked decrease in methane production rate 48 hr post-QAC addition compared to those with lower QAC levels. These data suggest a critical QAC level between 50 and 100 mg QAC/L for the thermophilic sludge. Previous studies observed inhibitory effects above 25 mg QAC/L for mesophilic methanogenic communities (Tezel et al., 2006, 2007). QAC concentration also affected the quality of biogas produced by sludge (Fig. 1c). The methane content of the biogas produced by sludge significantly decreased as QAC concentration increased ($p = 0.008$). These data indicate that methanogenesis in the thermophilic sludge was more sensitive to the concentration of QAC compared to upstream metabolic processes that produce carbon dioxide. A similar response was previously observed for mesophilic sludge (Tezel et al., 2006). Although some of these upstream processes, such as the production of acetate from other organic acids, produce gaseous hydrogen in tandem with carbon dioxide, no accumulation of hydrogen gas was detected for any treatment (data not shown). A significant negative correlation was observed between the final sludge pH and the QAC level ($p = 0.004$, Fig. 1d). However, the lowest pH measured (7.85) was still well within the tolerable range for anaerobic digestion (Cioabla et al., 2012).

2.2. Microbial community composition

Calculation of Good's coverage values predicted that over 99.9% of OTUs were accounted for in the sequencing data for each

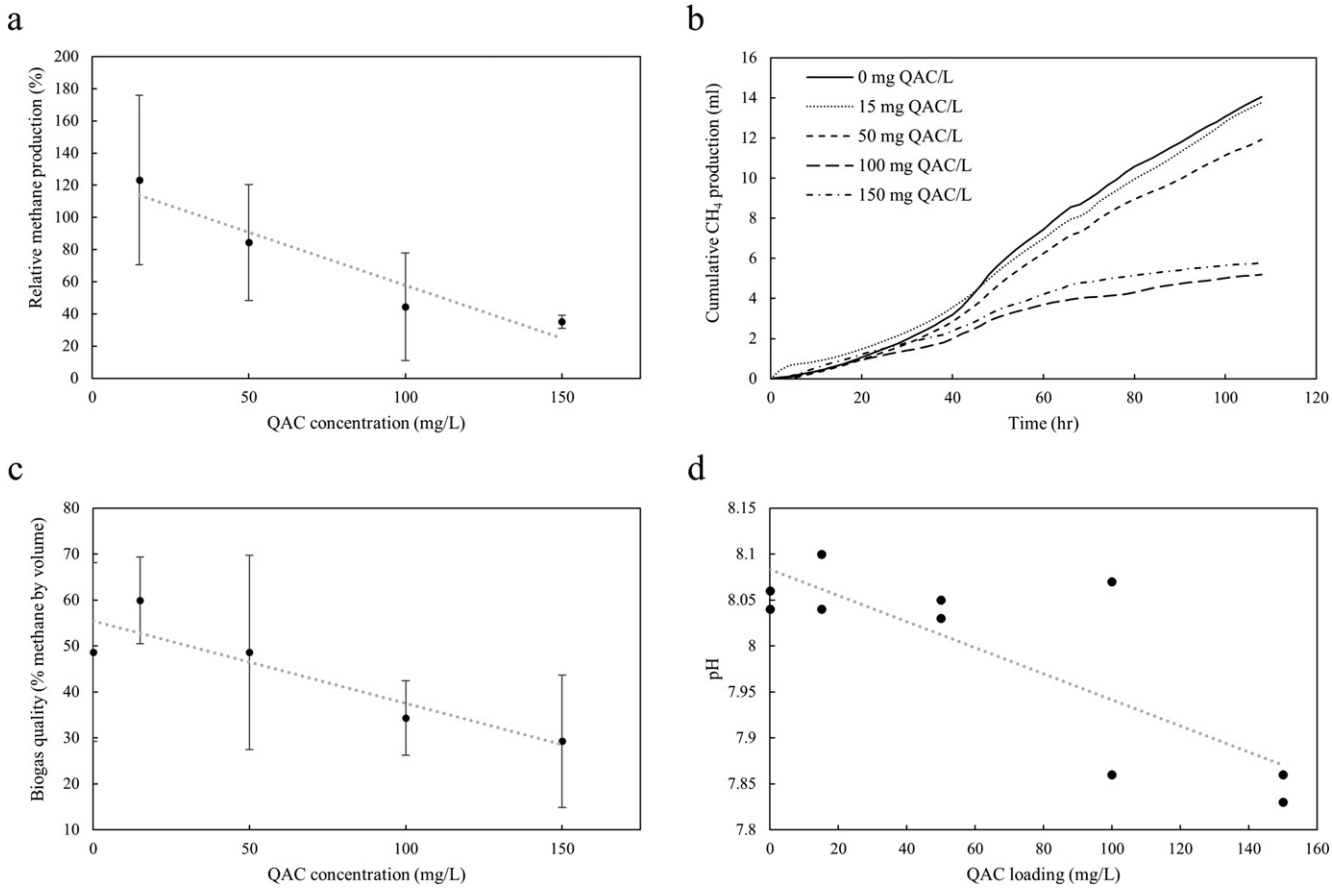

Fig. 1 – Biomethane production from thermophilic sludge containing varying levels of quaternary ammonium compounds (QACs). (a) Final cumulative methane production expressed as a percentage of that observed in control reactors lacking QAC. (b) Cumulative methane production over the first 4.5 days of culture. (c) Biogas quality estimated from cumulative production of methane and carbon dioxide over the culture period. (d) Sludge pH at the conclusion of the incubation. Dotted lines indicate the line of best fit for the data. Error bars represent one standard deviation. *n* = 4 for a-c, *n* = 2 for d.

microbial community analyzed. At the whole community level, diversity index (H′) did not show any significant difference in response to QAC level (Table 1). Separate analysis of archaea and bacteria within the communities revealed differing trends between these sub-communities in response to QAC contamination. Increasing QAC levels corresponded to a significant increase in dissimilarity for archaeal sub-communities compared to archaea in the sludge prior to treatment ($p = 0.009$, Fig. 2). Archaeal communities were represented by four genera: *Methanobacterium*, *Methanoculleus*, *Methanothermobacter*, and an uncharacterized genus within family *WCHD3-02* (class *Thermoplasmata*). *Methanoculleus* dominated archaeal communities across all treatments (relative abundance >83%, Fig. 3). However, its relative abundance significantly decreased ($p = 0.009$) in favor of *Methanothermobacter* and an OTU genus within family *WCHD3-02* at greater QAC concentrations. The abundance of *Methanoculleus* in all cultures suggested that this genus was likely responsible for most of the sludge methanogenic activity. *Methanoculleus* archaea are hydrogenotrophic methane producers (Barret et al., 2013; Wasserfallen et al., 2000). The prominence of hydrogenotrophic methanogens in all communities indicated that the community likely employed syntrophic acetate conversion, where non-methanogenic microorganisms within the sludge community oxidize acetate to produce CO_2 and H_2 for hydrogenotrophic methanogenesis. Syntrophic acetate oxidation is most thermodynamically favorable at elevated temperatures (Karakashev et al., 2006). As a result, the thermophilic communities studied here likely differ considerably from the mesophilic communities examined in prior QAC contamination studies (Tezel et al., 2006, 2007), which may have relied on other archaea and

Table 1 – Sequencing coverage and community diversity indicators for sludge communities exposed to varying levels of QAC. Values are presented as mean ± standard deviation (*n* = 4).

QAC level (mg/L)	Good's coverage	Shannon (H′)
0	$0.9997 \pm 5 \times 10^{-5}$	2.07 ± 0.07
15	$0.9996 \pm 5 \times 10^{-5}$	2.11 ± 0.09
50	$0.99975 \pm 5 \times 10^{-5}$	2.13 ± 0.05
100	$0.9996 \pm 5 \times 10^{-5}$	2.09 ± 0.11
150	$0.9997 \pm 5 \times 10^{-5}$	2.13 ± 0.11

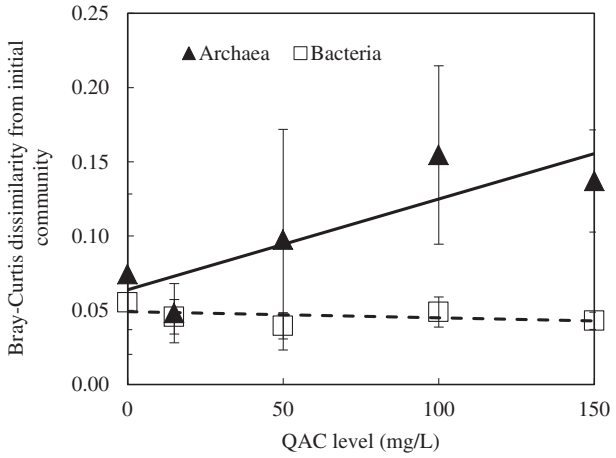

Fig. 2 – Bray–Curtis dissimilarity of bacteria and archaea within sludge communities exposed to QACs relative to the initial sludge community. Solid and dotted lines represent lines of best fit for archaeal and bacterial sub-communities, respectively. Error bars indicate one standard deviation. *n* = 4.

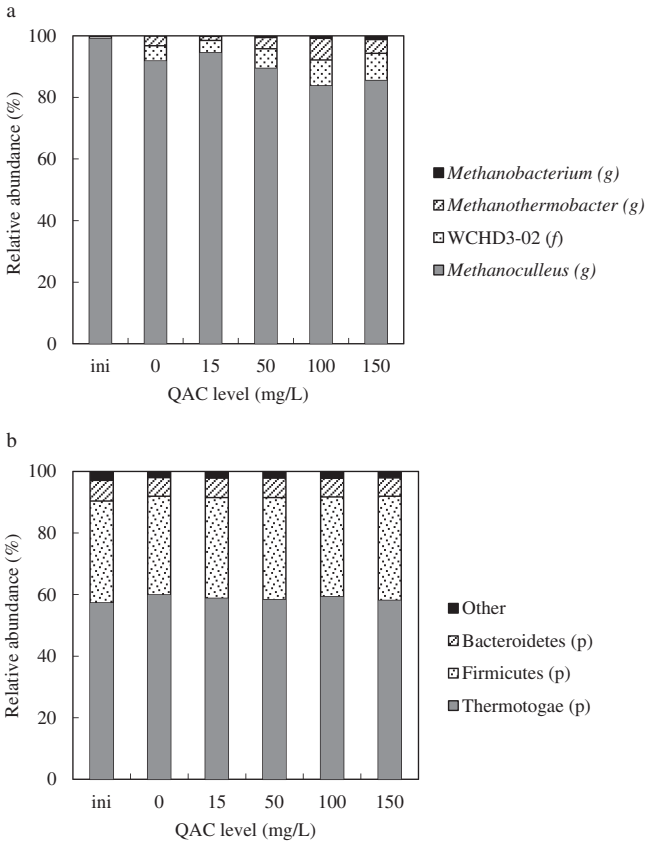

Fig. 3 – Phylogenetic composition of anaerobic digester microbial communities exposed to various levels of QAC sanitizer and the initial inoculum (ini). Data correspond to (a) archaea and (b) bacteria sub-communities. For clarity, archaea are presented at the lowest resolved phylogenetic classification while bacteria are presented at the phylum level (p, phylum; f, family; g, genus). *n* = 4.

methanogenic pathways that are more thermodynamically favorable at lower temperatures, such as acetotrophic methanogenesis. There may be innate differences in the sensitivity of thermophilic and mesophilic archaea to QACs. For instance, it has been observed that thermophilic archaea can be tolerant to a variety of other environmental stresses (Mesbah and Wiegel, 2012). The overall robustness of certain thermophilic archaea may contribute to the greater QAC tolerance observed in this study compared to previous research with mesophilic sludge communities. However, additional research is needed to separate other effects, such as differential adsorption of QACs to suspended solids in thermophilic and mesophilic sludges, that could also affect QAC availability and tolerance in sludges.

Although the culture duration used in this study was sufficient to elucidate differences in biogas production in response to QAC contamination, it was less than the 25 to 30 days hydraulic retention time typically used in anaerobic digesters. It is possible that the pH depression and archaeal restructuring observed at high QAC concentrations could become more drastic over time. Given their abundance within all archaeal sub-communities, *Methanoculleus* sensitivity to QACs is likely a major factor in the overall anaerobic digestion sensitivity to QAC contamination. Similar changes between *Methanoculleus* and other methanogenic archaea has been observed previously in response to digester perturbations (Lee et al., 2014).

Bacterial sub-communities showed no significant relationship between dissimilarity from the initial community state and QAC concentration ($p = 0.79$, Fig. 2). Sludge communities contained 20 bacterial phyla spanning 203 genera. The most abundant phyla, *Thermotogae*, *Firmicutes*, and *Bacteroidetes*, showed no significant changes in relative abundance for the QAC levels tested ($p = 0.442$, 0.212 and 0.592, respectively; Fig. 3). Within these phyla, twelve OTUs accounted for more than 87% of bacterial community abundance for all treatments (Appendix A Table S1). Three OTUs showed changes in relative abundance in response to varying QAC concentration: Clostridiales Family XI. Incertae Sedis family and *Tepidanaerobacter* genus increased with increasing QAC concentration whereas an OTU within the MBA08 order decreased as QAC levels increased. However, when the Bonferroni correction was used to account for multiple comparisons and establish a new critical *p*-value ($p \leq 0.0042$), corresponding to a familywise error rate of 0.05 across all OTUs, no OTUs showed significant changes in relative abundance in response to QAC concentration.

The differential response in archaeal and bacterial communities to increasing QAC concentration, as indicated by both phylogenetic restructuring and changes in biogas quality, is consistent with prior research that found that methanogenic archaea were more sensitive to ammonium concentration than sludge bacteria (Sawayama et al., 2004). The differing sensitivity to QAC may relate to physiological differences between certain archaea and bacteria, such as preference for different compatible solutes to manage osmotic stress (da Costa et al., 1998). Additionally, the unique lipids that archaea utilize to withstand thermophilic environments (van de Vossenburg et al., 1998)

may ultimately make them less tolerant of QACs. Additional research is needed to explore these possibilities.

3. Conclusion

This study suggests a negative impact of QAC on thermophilic digester performance. Further studies to confirm these effects on a larger scale and with a longer retention time are necessary. Data regarding the tolerance of anaerobic digestion microbial communities exposed to QAC sanitizer can inform digester operational procedures and waste treatment practices. QAC sanitizers are often recommended for use at levels up to 400 mg/L (F-29 sanitizer label), considerably greater than the inhibitory threshold for the thermophilic sludge community studied here. Therefore, treatment or dilution of sanitizer wastewater streams with significant QAC concentration will be required ahead of digester loading. Moreover, phylogenetic composition data from anaerobic digestion communities will be useful for predicting QAC susceptibility in other methanogenic communities.

Supplementary data to this article can be found online at doi:10.1016/j.jes.2016.10.005.

Acknowledgments

The authors thank CleanWorld Inc. for assistance with collecting digester sludge samples, Ryan Poon for assistance with digester maintenance and isolation of microbial genomic DNA, and Steven Singer of the Joint Bioenergy Institute and the Lawrence Berkeley National Laboratory for consultation in developing the study. This work was performed as part of the DOE Joint BioEnergy Institute (http://www.jbei.org) supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the U.S. Department of Energy. 16S rRNA gene sequencing was conducted by the Joint Genome Institute, which is supported by the Office of Science of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.

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