

# Enumeration of Organohalide Respirers in Municipal Wastewater Anaerobic Digesters

## Supplementary Issue: Water Microbiology

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**ABSTRACT:** Organohalide contaminants such as triclosan and triclocarban have been well documented in municipal wastewater treatment plants (WWTPs), but the degradation of these contaminants is not well understood. One possible removal mechanism is organohalide respiration by which bacteria reduce the halogenated compound. The purpose of this study was to determine the abundance of organohalide-respiring bacteria in eight WWTP anaerobic digesters. The obligate organohalide respiring *Dehalococcoides mccartyi* was the most abundant and averaged  $3.3 \times 10^7$  copies of 16S rRNA genes per gram, while the *Dehalobacter* was much lower at  $2.6 \times 10^4$  copies of 16S rRNA genes per gram. The genus *Sulfurospirillum* spp. was also detected at  $1.0 \times 10^7$  copies of 16S rRNA genes per gram. No other known or putatively organohalide-respiring strains in the *Dehalococcoidaceae* family were found to be present nor were the genera *Desulfotobacterium* or *Desulfomonile*.

**KEYWORDS:** triclosan, triclocarban, reductive dehalogenation, *Dehalococcoides mccartyi*, *Dehalobacter*

**SUPPLEMENT:** Water Microbiology

**CITATION:** Smith et al. Enumeration of Organohalide Respirers in Municipal Wastewater Anaerobic Digesters. *Microbiology Insights* 2015;8(S2) 9–14 doi:10.4137/MBI.S31445.

**TYPE:** Original Research

**RECEIVED:** August 4, 2015. **RESUBMITTED:** September 10, 2015. **ACCEPTED FOR PUBLICATION:** September 15, 2015.

**ACADEMIC EDITOR:** Raul Rivas, Editor in Chief

**PEER REVIEW:** Three peer reviewers contributed to the peer review report. Reviewers' reports totaled 511 words, excluding any confidential comments to the academic editor.

**FUNDING:** This work was supported by Oklahoma State University start-up funds to MJK. The authors confirm that the funder had no influence over the study design, content of the article, or selection of this journal.

**COMPETING INTERESTS:** Authors disclose no potential conflicts of interest.

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## Introduction

A wide variety of halogenated compounds (organohalides or organohalogens) are found in wastewater coming from a variety of sources such as cleaning agents, disinfectants, industrial runoff, and pesticides.<sup>1</sup> Compounds such as polychlorinated biphenyls, chlorophenols, triclocarban, triclosan, and diclofenac have been quantified through municipal wastewater treatment plants (WWTPs).<sup>2,3</sup> Because of the carcinogenic, toxic, and/or estrogenic nature of many of these compounds,<sup>4–6</sup> as well as the potential for the spread of antibiotic resistance by some such as triclosan,<sup>7</sup> the presence of these compounds in wastewater raises concerns for the land application of WWTP biosolids and downstream water quality.

In WWTPs, the removal of organohalides from the water varies; however, there is typically a reduction of the concentration throughout the system.<sup>8–11</sup> For example, triclosan was found in one WWTP to have an influent concentration of 0.93–2.11  $\mu\text{g L}^{-1}$ , and the effluent concentration was found to be 0.07–0.15  $\mu\text{g L}^{-1}$ .<sup>2</sup> A measurement of adsorbed organic halogen compounds showed a significant reduction as it passed through another WWTP.<sup>9</sup> Though much of the halogenated compounds are being

removed from the water during treatment, these compounds are predominately sorbed onto the solids and moved into solids treatment processes such as anaerobic digesters.<sup>11</sup> Some degradation of organohalides is found likely to occur from mass balances.<sup>11</sup> One study, for example, showed that 93% of triclosan is removed from the influent, with 41% sorbed to the sludge and 52% transformed or otherwise lost in the process.<sup>2</sup>

Though the concentrations of organohalides in WWTPs are becoming well documented,<sup>12</sup> the degradation of these compounds in WWTPs is not well understood. One possible removal mechanism is through organohalide respiration, where the organohalide is dehalogenated, and thus typically transformed to less toxic or nontoxic compounds.<sup>13</sup> A wide range of organohalide-respiring bacteria has been isolated and many of their genomes have been sequenced.<sup>14</sup> In a WWTP, these processes may occur in anaerobic digesters, where reducing conditions would be favorable to organohalide-respiring physiology. Indeed, the organohalide-respiring strain *Dehalococcoides mccartyi* st. 195 as well as others were isolated from enrichment cultures stemming from WWTP digesters.<sup>15–17</sup> Some of the contaminants in WWTPs such as polychlorinated biphenyls and chlorophenols are well known to be



degraded by organohalide respirers<sup>18,19</sup> and have been found to be dechlorinated in anaerobic digesters.<sup>9,20</sup> Dechlorination products of the two chlorinated compounds with the highest concentrations in WWTPs, triclocarban, and triclosan, have also recently been identified in sludge treatment processes,<sup>21</sup> and organohalide respiration of these compounds has been suggested by some studies,<sup>22</sup> but not yet conclusively proven.

The abundance and diversity of organohalide-respiring bacteria in WWTP anaerobic digesters have not been directly studied. A meta-analysis<sup>23</sup> of public database-deposited sequences from WWTP digesters and metagenomics of digesters<sup>24,25</sup> have produced scant insight toward the abundance of organohalide-respiring bacteria as well. The purpose of this study was to determine the abundance of organohalide-respiring bacteria in WWTP anaerobic digesters.

## Methodologies

Anaerobic digester sludge was collected from seven WWTPs in the central and northeastern Oklahoma region. One WWTP operated a two-stage anaerobic digestion process, and samples were collected from both the first- and the second-stage digester. Samples were collected in August 2014 in 1 L

plastic bottles that were filled, tightly capped, and transported to the laboratory within two hours, where they were placed in a refrigerator.

DNA was extracted from between 0.25 and 0.5 g of sludge using the PowerSoil DNA extraction kit (MoBio Laboratories) according to manufacturer's recommendations. Previously published quantitative PCR (qPCR) methodologies were used to quantify the known obligate organohalide-respiring genera of *D. mccartyi*,<sup>26</sup> the *Dehalobium chloroerca* DF-1/o-17 group,<sup>27</sup> *Dehalogenimonas*,<sup>28</sup> and *Dehalobacter*.<sup>29</sup> Also targeted were the facultative organohalide-respiring *Desulfitobacteria*,<sup>29</sup> *Geobacter*,<sup>30</sup> *Desulfomonile tiedjei*,<sup>31</sup> *Desulfovibrio*,<sup>32</sup> and *Sulfurospirillum*.<sup>33</sup> The total bacteria 16S rRNA genes were also quantified.<sup>34</sup> The putatively dechlorinating Gopher group<sup>35</sup> and a less specific *Dehalococcoides*-like *Chloroflexi* group<sup>36</sup> were also attempted for amplification. The primers for each qPCR protocol are listed in Table 1. Each qPCR mixture totaled 10 µL and contained 1 × iTaq SYBR Green Supermix with Rox (Bio-Rad Laboratories), 300 nM of each primer, and 0.5 µL of undiluted DNA extract or standard. Primers were purchased from Life Technologies. Analysis was on a CFX Connect Real Time System with

**Table 1.** Primers used to study organohalide-respiring organisms and Bacteria.

| PHYLOGENETIC TARGET                        | PRIMER | SEQUENCE                             | REFERENCE |
|--|--------|--------------------------------------|-----------|
| <i>Dehalococcoides mccartyi</i>            | 582F   | 5'-CTGTTGGACTAGAGTACAGC-3'           | 26        |
|  | 728R   | 5'-GTGACAACCTAGAAAACCGCCTT-3'        |           |
| "Dehalococcoides-like <i>Chloroflexi</i> " | 1150F  | 5'-GGGCTACACACACGCTACAATGG-3'        | 36        |
|  | 1286R  | 5'-GATATGCGGTTACTAGCAACTCCAAC-3'     |           |
| <i>Dehalobium chloroerca</i> DF1           | 866F   | 5'-CGCTTTAAGTRTCCCGCC-3'             | 27        |
|  | 1265R  | 5'-CCTATTGCTACCTGCTGTACC-3'          |           |
| <i>Dehalogenimonas</i> spp.                | 634F   | 5'-GGTCATCTGATACTGTTGGACTTGAGTATG-3' | 28        |
|  | 799R   | 5'-ACCCAGTGTTTAGGGCGTGGACTACCAGG-3'  |           |
| <i>Dehalobacter</i> spp.                   | 447F   | 5'-GATTGACGGTACCTAACGAGG-3'          | 29        |
|  | 647R   | 5'-TACAGTTTCCAATGCTTTACGG-3'         |           |
| <i>Desulfitobacterium</i> spp.             | 406F   | 5'-GTACGACGAAGGCCTTCGGGT-3'          | 29        |
|  | 619R   | 5'-CCCAGGGTTGAGCCCTAGGT-3'           |           |
| <i>Geobacter lovleyi</i>                   | 564F   | 5'-AAGCGTTGTTCCGAWTTAT-3'            | 30        |
|  | 840R   | 5'-GGCACTGCAGGGGTCAATA-3'            |           |
| <i>Desulfomonile</i> spp.                  | 205F   | 5'-GGGTCAAAGTCGGCCTCTCGACG-3'        | 31        |
|  | 628R   | 5'-GCTTTCACATTCGACTTATCG-3'          |           |
| <i>Desulfovibrio</i> spp.                  | 691F   | 5'-CCGTAGATATCTGGAGGAACATCAG-3'      | 32        |
|  | 826R   | 5'-ACATCTAGCATCCATCGTTTACAGC-3'      |           |
| <i>Sulfurospirillum</i> spp.               | 114F   | 5'-GCTAACCTGCCCTT TAGTGG-3'          | 33        |
|  | 421R   | 5'-GTTTACACACCGAAATGCGT-3'           |           |
| Gopher Group                               | 163F   | 5'-TGACCYTGCCATCAGGGA-3'             | 35        |
|  | 441R   | 5'-TATTTTACAACCCGAAGGCCTTCG-3'       |           |
| Bacteria                                   | 341F   | 5'-CCTACGGGAGGCAGCAG-3'              | 34        |
|  | 534R   | 5'-ATTACCGCGGCTGCTGGC-3'             |           |

**Table 2.** Parameters of qPCR analysis.

| PHYLOGENETIC TARGET                                 | EFFICIENCY, $R^2$ | LINEAR RANGE<br>(COPIES PER REACTION) | QUANTIFICATION LIMITS<br>(COPIES PER g) |
|---|-------------------|---------------------------------------|---|
| <i>Dehalococcoides mccartyi</i>                     | 104%, 0.99        | $1.9 \times 10^1$ – $1.9 \times 10^6$ | 7,600                                   |
| " <i>Dehalococcoides</i> -like <i>Chloroflexi</i> " | 105%, 0.99        | $1.9 \times 10^2$ – $1.9 \times 10^8$ | 76,000                                  |
| <i>Dehalogenimonas</i> spp.                         | 93%, 0.99         | $1.7 \times 10^1$ – $1.7 \times 10^7$ | 6,800                                   |
| <i>Dehalobacter</i> spp.                            | 110%, 0.99        | $1.2 \times 10^1$ – $1.2 \times 10^5$ | 4,800                                   |
| <i>Desulfitobacterium</i> spp.                      | 110%, 0.97        | $2.2 \times 10^2$ – $2.2 \times 10^9$ | 88,000                                  |
| <i>Geobacter lovleyi</i>                            | 76%, 0.98         | $5.8 \times 10^1$ – $5.8 \times 10^6$ | 23,200                                  |
| <i>Desulfovibrio</i> spp.                           | 99%, 0.98         | $1.3 \times 10^2$ – $1.3 \times 10^9$ | 5,200                                   |
| <i>Sulfurospirillum</i> spp.                        | 87%, 0.99         | $6.7 \times 10^1$ – $6.7 \times 10^6$ | 26,800                                  |
| <i>Bacteria</i>                                     | 75%, 0.96         | $1.9 \times 10^5$ – $1.9 \times 10^9$ | $7.6 \times 10^8$                       |

CFX Manager software (Bio-Rad Laboratories). For all phylogenetic targets other than *D. mccartyi*, the thermocycling protocol was 95°C for three minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. For *D. mccartyi*, the thermocycling protocol was 95°C for 3 minutes followed by 40 cycles of 95°C for 15 seconds and 56°C for 30 seconds. A melting curve analysis was performed after each complete run to ensure that primer-dimers were not amplified and that the amplification was specific. Standards for each qPCR were prepared from plasmid extracts containing the 16S rRNA gene of interest and quantified from the QuantiFluor dsDNA dye system (Promega Corporation). Each sample and standard was analyzed in triplicate qPCR runs. Each qPCR analysis was performed with negative controls (water blanks) and positive controls (DNA extract from mixed cultures with the targeted 16S rRNA gene).

The primers, amplification efficiencies, standard curve  $R^2$  values, and standard ranges are listed in Table 2. Standard ranges represent standards between the lowest reproducible serially diluted standard and the highest standard in the linear range. Quantification limits are calculated to be the minimum concentration of 16S rRNA genes needed to be detected above the minimum standard. The *D. chlorochoercia* DF-1/o-17 group, Gopher group, and *Desulfomonile* were found not to be amplified from any digester samples under a range of melting temperatures (50°C–62°C), and standard curves were not produced for these methods. Furthermore, *Desulfitobacterium* were not detected above the quantification limits for any sample. For all genera that were above the quantification limit in the samples, clone libraries of the product were produced using the pGEM-T Easy Vector system (Promega) from a minimum of two samples to verify specificity of the amplification. All sequences from clone libraries from the *Dehalobacter*, *D. mccartyi*, and *Sulfurospirillum* amplification were verified to be closely related to their targeted genera (>98% sequence identity) with a total of 12, 9, and 9 clones, respectively. Amplifications were found to be unspecific for *Dehalogenimonas* (9 total clones, all related to *Bacteroidetes*

and *Dehalococcoides*-like *Chloroflexi* (10 clones, 9 from various *Firmicutes* and 1 from the class *Anaerolineae* in *Chloroflexi*). The amplifications of *Geobacter* as well as *Desulfovibrio* yielded amplicons with a mixture of targeted and nontargeted genera with 6 out of 16 clones being specific to their targeted genus (>98%) for *Geobacter* and 2 out of 13 clones being specific for the *Desulfovibrio* amplification. The nontargeted *Geobacter* amplicons were from various genera in the *Proteobacteria*, while the nontargeted *Desulfovibrio* amplicons were *Clostridia* from the phylum *Firmicutes* and various genera of *Anaerolineae* from the *Chloroflexi* phylum.

Terminal restriction fragment length polymorphism (TRFLP) was used in this study to identify nontargeted strains of the *Dehalococcoidaceae* family of *Chloroflexi*,<sup>37</sup> which may contain nonisolated strains of organohalide-respiring bacteria. The PCR amplification and enzyme digestion were performed as described by Krzmarzick et al.,<sup>36</sup> except that the general *Eubacteria* primer 8F (5'-AGAGTTTGATCMTG GCTCAG-3') was used in lieu of the primer Dhc553F. The fragment-size analysis of amplifications was performed at the DNA/Protein Core facility at Oklahoma State University with an ABI Model 3730 Analyzer with a MapMarker1000 size standard (BioVentures, Inc.). Clone libraries were performed on the PCR amplicons of the anaerobic digester samples using the pGEM-T Easy Vector system (Promega) and sequenced at the DNA/Protein core facility at Oklahoma State University with an ABI Model 3730 Analyzer. A total of 17 unique sequences were found, which corresponded to 15 OTUs (theoretical digestion sizes) representing all major groups (>1% of any sample's total peak area) found in the TRFLP analysis in the samples. BLAST analysis of these sequences indicated that none of these sequences belonged to the *Chloroflexi*. Thus, any putative reductive dechlorinating *Chloroflexi* was of low abundance compared with the nontargeted groups that were amplified.

## Results and Discussion

*Dehalogenimonas* spp., *D. chlorochoercia* DF-1, strain o-17, *Desulfitobacterium* spp., the Gopher group, *D. tiedjei*, and nonisolated



**Table 3.** Number of 16S rRNA genes of Bacteria and organohalide respirers in WWTP anaerobic digesters (logarithmic units per gram of digester contents).

| SAMPLE     | Bacteria                 | Dehalobacter     | Dehalococcoides <sup>1</sup> | Sulfurospirillum | Geobacter    | Desulfovibrio |
|------------|--------------------------|------------------|------------------------------|------------------|--------------|---------------|
| Digester 1 | 11.4 ± 0.04 <sup>2</sup> | 4.45 ± 0.23      | 7.28 ± 0.02                  | 6.92 ± 0.13      | <7.44 ± 0.19 | <7.56 ± 0.02  |
| Digester 2 | 11.1 ± 0.07              | 3.85 ± 0.10      | 7.14 ± 0.01                  | 6.52 ± 0.05      | <7.23 ± 0.11 | <7.38 ± 0.01  |
| Digester 3 | 11.7 ± 0.03              | 4.04 ± 0.15      | 7.72 ± 0.02                  | 7.33 ± 0.06      | <8.03 ± 0.09 | <7.39 ± 0.09  |
| Digester 4 | 11.3 ± 0.01              | 4.29 ± 0.08      | 7.55 ± 0.02                  | 7.38 ± 0.09      | <7.83 ± 0.10 | <7.40 ± 0.05  |
| Digester 5 | 11.1 ± 0.12              | 4.24 ± 0.23      | 7.57 ± 0.09                  | 6.97 ± 0.09      | <7.49 ± 0.32 | <7.34 ± 0.09  |
| Digester 6 | 11.2 ± 0.08              | 4.62 ± 0.06      | 7.52 ± 0.09                  | 6.73 ± 0.03      | <7.49 ± 0.09 | <8.39 ± 0.01  |
| Digester 7 | 11.2 ± 0.24              | 4.77 ± 0.03      | 7.33 ± 0.05                  | 6.55 ± 0.05      | <7.41 ± 0.53 | <7.15 ± 0.05  |
| Digester 8 | 11.6 ± 0.07              | BDL <sup>3</sup> | 7.69 ± 0.07                  | 6.80 ± 0.17      | <7.70 ± 0.11 | <7.45 ± 0.08  |

**Notes:** <sup>1</sup>*D. mccartyi* determined from 582F//728R primer pair in Table 1. <sup>2</sup>Mean ± standard deviation of qPCR analysis. <sup>3</sup>Below detection limit.

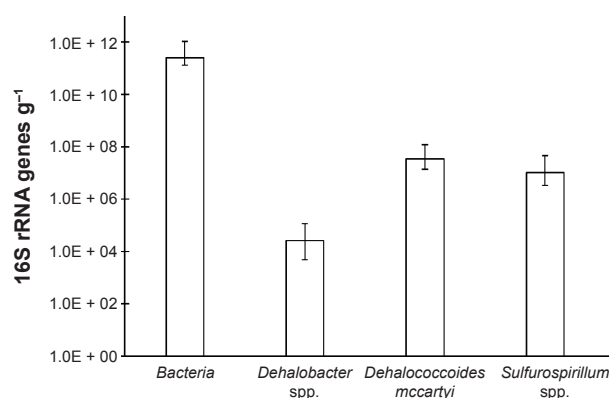
and putatively organohalide-respiring strains in the *Dehalococcoidaceae* family were not found to be present in the qPCR and TRFLP methods used in this study in any of the sampled digesters. The amplification of nontargeted groups in the qPCR results for *Dehalogenimonas* and the *Dehalococcoides*-like *Chloroflexi* and the dominance of nontargeted groups in the TRFLP analysis preclude a conclusive determination that these organisms are not in digesters. These bacteria may still be present at some level but low in comparison to the nontargeted organisms that were amplified. For example, the species *D. mccartyi* was neither recovered in the TRFLP analysis nor in the clone libraries from the *Dehalococcoides*-like *Chloroflexi* qPCR analysis, though its 16S gene has 100% identities to the primers used in those methods. Yet the more specific qPCR method targeting this genus and clone libraries of this method verified its existence.

In a meta-analysis of sequences deposited in public databases, 12 sequences related to *Dehalogenimonas* spp. (out of >16,500 total sequences) were identified with anaerobic digester communities,<sup>23</sup> indicating *Dehalogenimonas* spp. may be present in low levels of anaerobic digesters. The strains *Dehalobium* DF-1, o-17, *Desulfitobacteria* spp., Gopher group, and *Desulfomonile* may similarly be present in anaerobic digesters though not found in this study; indeed, *D. tiedjei* was isolated from anaerobic digester sludge.<sup>38</sup>

The *Desulfovibrio* and *Geobacter* genera were detected in the anaerobic digester samples. The quantification of *Desulfovibrio* ranged from  $1.4 \times 10^7$  to  $2.4 \times 10^8$  copies of 16S rRNA genes per gram, while the *Geobacter* ranged from  $1.7 \times 10^7$  to  $1.1 \times 10^8$  copies of 16S rRNA genes per gram (Table 3). These amplifications must be interpreted as maximal values as clone library analysis of their products indicated nontargeted genera were amplified as well. Furthermore, the sequences recovered from these amplifications that were closely related to their targeted genera were still <98% identical to the 16S rRNA genes of *Geobacter lovleyi*<sup>30</sup> and *Desulfovibrio dechloracetivorans*<sup>39</sup> strains, respectively, which are the facultative organohalide-respiring strains of their respective genera. Thus, the amount of

these genera capable of organohalide respiration in anaerobic digesters may be minimal.

The quantification of *D. mccartyi*, *Dehalobacter* spp., *Sulfurospirillum* spp., and total bacteria are shown in Table 3 and Figure 1. The quantification of *D. mccartyi*, *Dehalobacter* spp., and *Sulfurospirillum* spp. are confirmed with clone library analysis of their amplification products. Total bacteria 16S rRNA genes in the anaerobic digesters ranged from  $1.3 \times 10^{11}$  to  $5.4 \times 10^{11}$  copies of 16S rRNA genes per gram with an average of  $2.5 \times 10^{11}$  16S rRNA genes per gram. The organohalide-respiring genera were orders of magnitude less. *Dehalobacter* was detected just over the quantification limit in seven digesters while being below the quantification limit in one digester. *Dehalobacter* ranged up to  $5.9 \times 10^4$  copies of 16S rRNA genes per gram with an average of  $2.6 \times 10^4$  copies of 16S rRNA genes per gram in the seven digesters with *Dehalobacter*. The obligate organohalide-respiring *D. mccartyi* was more abundant and ranged from  $1.4 \times 10^7$  to  $5.2 \times 10^7$  copies of 16S rRNA genes per gram with an average of  $3.3 \times 10^7$  copies of 16S rRNA genes per gram. The genera containing the facultative organohalide-respiring bacterium



**Figure 1.** The averages and ranges of 16S rRNA genes per gram quantified with qPCR in eight anaerobic digester samples for bacteria, *Dehalobacter* spp., *D. mccartyi*, and *Sulfurospirillum* spp.





*Sulfurospirillum multivorans*<sup>40</sup> ranged from  $3.3 \times 10^6$  to  $2.4 \times 10^7$  copies of 16S rRNA genes per gram and averaged  $1.0 \times 10^7$  copies of 16S rRNA genes per gram. *Sulfurospirillum* clones were more closely related to strains other than *S. multivorans*, which is the strain found to have reductive dehalogenase genes.<sup>40</sup> The percent identity with *S. multivorans* was typically 96%–97%, while other *Sulfurospirillum* strains were related at up to 100% identity. Thus, these measurements may be true for the genus; they likely overestimate greatly the abundance of *Sulfurospirillum* capable of organohalide respiration.

Despite concentrations of organohalides ranging in the mg/kg levels in anaerobic digester sludge,<sup>11</sup> the microbial communities capable of organohalide respiration are relatively minimal, representing about 0.02% of the total bacterial community. Though low levels of microbial communities can have relevant implications for degradation, these levels of bacteria are rather low and may be a major contributing reason that the degradation of organohalide contaminants are limited. The differences of populations between digesters were less than one order of magnitude indicating that these populations may not vary significantly between WWTP anaerobic digesters operated in the United States. This small range in organohalide-respiring populations is in contrast to the several orders of magnitude range of organohalides found in anaerobic digesters.<sup>12</sup> The concentrations of organohalides were not found in the digesters of this study, and thus, the relationship between organohalide abundance and organohalide-respiring populations is not known. Digesters 1 and 2 in Table 3 are, respectively, the primary and secondary digesters from a single WWTP. The secondary digester contained lower abundances than the primary digester for all organohalide-respiring bacteria and was generally on the low end of the ranges for all digesters studied.

The presence of obligate organohalide-respiring bacteria with broad dechlorination abilities<sup>14</sup> such as *Dehalococcoides* and *Dehalobacter* is significant as it does indicate that dechlorination processes do occur in WWTPs, and this process is likely responsible for some of the decrease in organohalides as suggested by the discovery of triclosan and triclocarban dechlorination products.<sup>21</sup> Though many conditions in digesters seem favorable for organohalide respirers, such as mesophilic temperatures<sup>41</sup> and highly reducing conditions,<sup>42</sup> other physiological conditions may be limiting their growth. The goal of reducing organohalides, however, may ultimately compete with (or complement) other goals in anaerobic digestion, such as increasing methane production.<sup>43</sup> Still, further work is warranted to determine if operational conditions may be adjusted to favor organohalide respiration to reduce the concentrations of these chemicals in biosolids.

### Author Contributions

Taught the study, which was a component of a graduate level course on molecular methods at Oklahoma State University,

collected samples, and conceived and designed the experiments: MJK. Performed experiments, analyzed data, and jointly developed the structure and arguments of the manuscript: BJKS, MAB, BAF, TML, RKR, and MJK. Wrote the first draft of the manuscript: BJKS. Made critical revisions: MJK. All authors reviewed and approved the final manuscript.

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