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Acetate promotes microbial reductive debromination of tetrabromobisphenol A during the startup phase of anaerobic wastewater sludge bioreactors

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Abstract

The detection of increasing concentrations of tetrabromobisphenol A (TBBPA) in wastewater treatment plants is raising concerns as TBBPA has been identified as a potentially toxic flame retardant. The objectives of this study were to evaluate the effect of acetate biostimulation on TBBPA microbial reductive debromination, and the response of anaerobic sludge associated microbial communities repeatedly exposed to TBBPA. Results indicate that the bulk of the microbial community did not experience significant shifts as a result of TBBPA exposure, and that only a small fraction of the community responded to the presence of TBBPA. Taxa most likely responsible for TBBPA transformation affiliated to Clostridiales and the wastewater sludge group Blvii28. The biostimulating effect of acetate was only observed during reactor startup, when acetogenesis was likely not yet occurring. However, when acetate likely started to be microbially generated in the reactor, acetate addition resulted in a slight but significant inhibiting effect on TBBPA transformation. A significant increase of hydrogenotrophic methanogens in the TBBPA-spiked reactor overtime implies that TBBPA degraders were not in direct competition with methanogens for H2. These results strongly suggest that TBBPA degrading taxa might have been primarily using acetate as an electron donor for the reductive debromination of TBBPA.

Keywords

Tetrabromobisphenol A; Flame retardant; Microbial reductive debromination; Anaerobic sewage sludge; Acetate biostimulation

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1. Introduction

Tetrabromobisphenol A (TBBPA) is one of the most widely used brominated flame retardant worldwide, and is primarily incorporated into electric and electronic products (e.g., computers, televisions, cellphones) in order to lower their flammability (Choi et al., 2009; Covaci et al., 2009). Although not considered acutely toxic, TBBPA has been shown to trigger toxicological responses in wildlife (Chen et al., 2016), and humans (Lai et al., 2015). Due to its structural resemblance to the thyroid precursor thyroxine, TBBPA has the potential to disrupt thyroid and estrogen regulatory functions, damage liver and kidney tissue, and increase risks of uterine cancer in mammals (Dunnick et al., 2015). The increasing occurrence of TBBPA in environmental samples, including aquatic sediments, agricultural soils, indoor dust, and wastewater sludge (Liu et al., 2016; Wang et al., 2015), is of growing concern as it suggests that exposure to TBBPA is likely common. TBBPA concentrations as high as 1,329 ng/g dw have been reported in sewage sludge, and effluents from wastewater treatment plants (WWTPs) still contain measurable levels of TBBPA (Liu et al., 2016), indicating that effluent discharge and biosolids land application are possible routes by which TBBPA enters the environment.

Microbial degradation is a major process of TBBPA transformation in the environment (Chen et al., 2015). Although complete biodegradation of TBBPA under continuous aerobic conditions can be achieved (An et al., 2011; Chang et al., 2012), a sequential process comprising of an anaerobic step in which TBBPA is first transformed to bisphenol A (BPA) *via* reductive microbial debromination, followed by an aerobic step in which BPA is rapidly mineralized has proven to substantially improve degradation rates compared to a transformation occurring exclusively under aerobic conditions (Liu et al., 2013; McAvoy et al., 2016; Ronen and Abeliovich, 2000). Microbial taxa responsible for the anaerobic phase of this process appear to be ubiquitous in anoxic habitats (Chang et al., 2012; Liu et al., 2013; McAvoy et al., 2016; Ravit et al., 2005; Yang et al., 2015), and several strains have been isolated from wastewater sludge (Peng et al., 2014; Peng et al., 2013; Wang et al., 2013; Zhang et al., 2013). Therefore, microbial community management strategies, whose underlying concept is to trigger a compositional shift towards a microbial community dominated by taxa with high degradative capabilities, are pertinent approaches to implement for improving *in situ* TBBPA biodegradation. Although bioaugmentation with TBBPA-degrading strains (Li et al., 2016), biostimulation with various substrates, including carbonaceous charred amendments (Lefèvre et al., 2018), Fe-based particles (Peng et al., 2017a), carbon sources (Chang et al., 2012; Peng and Jia, 2013; Zu et al., 2014), and domestication of the indigenous microbial community (Peng et al., 2017b) have been shown to enhance TBBPA microbial degradation, in most cases a comprehensive characterization of the microbial communities involved has not been performed. As a result of the growing demand for consumer electronics, TBBPA concentrations are expected to increase in the near future, in particular in WWTPs. Thus, evaluating the response of wastewater sludge microbial communities to TBBPA exposure, and identifying the microbial taxa responsible for TBBPA reductive debromination is extremely relevant, especially for the development of efficient microbial community management strategies that improve TBBPA *in situ* bioremediation. The objectives of this study were to: 1) evaluate biostimulation with acetate,

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a substrate previously shown to significantly increase TBBPA reductive debromination, (Lefevre et al., 2016; Peng and Jia, 2013), and can serve as a preferred electron donor for the complete degradation of other halogenated compounds (He et al., 2002; Lee et al., 2007); 2) determine if repeated exposure to TBBPA, i.e. domestication, can trigger a shift of the microbial community composition towards the dominance of a few taxa to whom TBBPA degradation can be attributed, as previously suggested (Peng et al., 2017b); and 3) assess the potential adverse impacts of TBBPA contamination on microbial communities essential to anaerobic wastewater treatment, particularly methanogens, which through their activity have been shown to promote TBBPA degradation (Iasur-Kruh et al., 2010; Voordeckers et al., 2002). To this end, bench-scale anaerobic sewage sludge bioreactors subjected to repeated exposure to TBBPA were operated and monitored. TBBPA and BPA concentrations were periodically measured using liquid chromatography with tandem mass spectrometry (LC-MS/MS), and microbial community composition and archaeal population dynamics were characterized using MiSeq Illumina sequencing, and quantitative PCR, respectively.

2. Material and methods

2.1. Reactor operation and sampling

Four bench scale anaerobic sludge reactors were assembled and operated as previously described (Lefèvre et al., 2018), and filled with 1.5 L of activated sludge (4.1±0.7 g/L of suspended solids) collected the same day at the North Durham WWTP (NC, US). The co-metabolic set of reactors (i.e., reactor #1 and #2) received 1 g of sodium acetate trihydrate (5 mM final concentration), while the metabolic set (i.e., reactor #3 and #4) did not receive any additional source of carbon. TBBPA reactors for each set (i.e., #1 and #3) were spiked with 15 mL of 0.4 g/L TBBPA stock solution for a target final concentration of 7.3 μM TBBPA, and control reactors (i.e., #2 and #4) received 15 mL of HPLC-grade acetone (solvent used to prepare the TBBPA stock solutions). Over the course of the experiment, which lasted 133 days, reactors received four additional spikes of TBBPA, acetone, and sodium acetate trihydrate identical to that just described. The first additional spike occurred when nearly all TBBPA had been depleted, which occurred at Day 41 in the TBBPA co-metabolic reactors and Day 55 for the metabolic set. The next three additional spikes occurred at Days 70, 83, and 104 for all reactors (Fig. 1). Triplicate samples of 2.5 and 1.5 mL of sludge (i.e., 12 mL total) were collected at 18 distinct time points for chemical and microbial analyses, respectively. The collection times consisted of Days 0 [before the first TBBPA spike], 3, 6, 11, 17, 24, 34, 41 [before the second spike of the co-metabolic reactor], 55 [before the second spike of the metabolic reactor], 70 [before the third spike], 83 [before and after the fourth spike], 88, 104 [before and after the fifth spike], 111, 126 and 133). Sludge samples for chemical analyses were stored at 4°C and processed within a day using LC-MS/MS to determine TBBPA and BPA concentrations in the sludge as previously described (Lefevre et al., 2016). All sludge samples collected from the TBBPA reactors (i.e., #1 and #3) were processed for chemical analyses. Sludge samples collected from the control reactors (i.e., #2 and #4) were only analyzed for Days 0, 3, 6, 17, 24, 34, 41, and 55, and revealed a TBBPA and BPA average background concentration of 0.04±0.07 and 0.03±0.02 μM, respectively. Sludge samples for microbial analyses were stored at −80°C, and a subset of 54 triplicate samples, collected on Days 0, 11, 24, 34, 41, 55, 70, 88, and 111 from the TBBPA and

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control metabolic reactors (i.e., #3 and #4), were processed for microbial genomic DNA extraction using the MoBio PowerSoil DNA isolation kit (MoBio, Carlsbad CA, US) as previously described (Lefevre et al., 2016).

2.2. Microbial community analyses

2.2.1 Illumina MiSeq sample preparation—Among the 54 samples processed for microbial DNA extraction, a subset of 36 triplicate samples collected on Days 0, 41, 55, 70, 88, and 111 from the TBBPA and control metabolic reactors were included in the Illumina MiSeq analysis. The Illumina library was prepared as previously described (Lefèvre et al., 2018). QIIME was used to analyze the Illumina MiSeq reads generated, and a workflow similar to the one previously described (Lefèvre et al., 2018) was followed. Briefly, after read filtering, a total of 5,821,575 sequences were obtained. Sequences were clustered together in operational taxonomic units (OTUs) with a similarity cutoff of 97%, and an additional filtering step removing OTUs having a relative abundance below 0.004% across samples was applied (justification for the selected threshold is shown in Fig. S1). This additional filtering step left 2,229,699 reads that clustered into 1,331 OTUs. OTUs taxonomic assignment was performed using the RDP classifier against the Greengenes database released in August 2013. Microbial community analyses were performed using a weighted UNIFRAC matrix-based non-metric multidimensional scaling (nMDS). An analysis of similarity (ANOSIM) was performed in order to test for the effect of time and TBBPA exposure (TBBPA *vs.* control) on microbial community composition dynamics.

2.2.2 Quantitative PCR assays—Seven qPCR assays targeting the *mcrA* gene (mcrA_1035F/mcrA_1530R primer set; (Pereyra et al., 2010), archaeal and bacterial 16S rDNA (ARC_787F/ARC_1059R and BAC_338F/BAC_805R primer sets, respectively; (Yu et al., 2005), and 16S rDNA of the archaeal orders Methanococcales, Methanobacteriales, Methanomicrobiales, and Methanosarcinales (MCC_495F/MCC_832R, MBT_857F/MBT1196R, MMB_282F/MMB_832R, MSL_812F/MSL_1159R primer sets, respectively; (Yu et al., 2005) were performed on the 54 sludge samples processed for DNA extraction. The 20 µl-qPCR reactions consisted of 20 to 50 ng of genomic DNA, for the *mcrA*, ARC, MCC, MBT, MMB, and MSL assays, and 0.2 ng of genomic DNA for the BAC assay, 200 nM of each primer and 1 X of the iTaq Universal SYBR Green Supermix (BioRad Laboratories, Hercules, CA, US). PCR amplifications were performed on the CFX96 Touch™ Real-Time PCR Detection System (BioRad Laboratories). For all qPCR assays, optimal PCR conditions started with an initial denaturation of 3 min at 95°C, followed by 40 cycles of 30 sec denaturation at 95°C, annealing of 30 sec at 60°C, and elongation of 30 sec at 68°C. A melting curve analysis, consisting of a gradual increase of temperature from 55 to 95°C, was immediately performed following each assay, and confirmed the absence of primer dimers and non-specific PCR products. Purified pCR2.1-TOPO vectors containing the DNA fragment targeted by each qPCR assays were used to generate standard curves. These plasmids were obtained from a previous study conducted in our laboratory on anaerobic reactors (unpublished), where each targeted fragment was amplified using the conditions used in this study, and cloned using the TOPO TA cloning kit with TOP10 chemically competent cells (Life Technologies, Carlsbad, CA, USA). Results were expressed in copies/mL of wet sludge and a series of t-tests was performed for each time

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point analyzed in order to detect significant ($p<0.05$) differences between the TBBPA and control metabolic reactors.

3. Results and discussion

3.1. TBBPA degradation

Microbial transformation of TBBPA to BPA has been shown to occur under anaerobic conditions with no further degradation of BPA (Ronen and Abeliovich, 2000). Therefore, if anaerobic microbial debromination of TBBPA takes place, the final molar quantity of BPA accumulated as a result should theoretically be equivalent to the molar quantity of TBBPA initially added. In the present study, by the end of the 133-day experiment, of the 51.5 μmol of TBBPA added (cumulative amount), 91.2±1.2 % and 86.3±2.2% had been transformed to BPA, in the metabolic and co-metabolic TBBPA-spiked reactors, respectively (Fig 1). Based on this observation, as well as the published results from two previous studies (Lefèvre et al., 2018; Lefevre et al., 2016), we concluded that microbial reductive debromination was the major process responsible for TBBPA transformation in our reactors. Unlike what was observed in our previous similar study in which acetate addition delayed the onset of TBBPA degradation by approximately two weeks (Lefevre et al., 2016), in the present study, TBBPA transformation started nearly immediately in both reactors with no acclimation period (Fig. 1). Such differences in the effect of acetate addition on the onset of TBBPA degradation might be attributed to the fact that the functional and taxonomic composition of the microbial communities in the sludge used to seed the reactors differed from one study to the other. Nevertheless, the nearly complete transformation of the first TBBPA spike was achieved significantly ($p<0.05$) earlier in the reactor amended with acetate (Fig. 1). Indeed, while almost all TBBPA added with the first spike had been transformed (96.4±0.7%) in the co-metabolic reactor by Day 34, in the metabolic reactor, 20 more days were necessary to reach a similar degree of TBBPA transformation (95.1±0.3% by Day 55). A closer look at the degradation patterns of the first TBBPA spike indicates that although TBBPA was initially transformed at similar rates in both reactors, at Day 17, TBBPA transformation in the metabolic reactor stalled for a few days and finally resumed at Day 34 (Fig. 1). In the presence of ;acetate though, TBBPA was continuously and steadily transformed. Although degradation patterns of the first TBBPA spike were significantly different between metabolic and co-metabolic reactors, for each individual following spikes, no apparent differences in degradation rates could be observed. However, such an effect might be hidden by the low frequency at which samples were collected, especially because the additional TBBPA spikes were degraded much faster than the initial spike. In fact, the quantity of TBBPA (cumulative amount) transformed to BPA by the end of the experiment was significantly higher in the metabolic than in the co-metabolic reactor (i.e., 91.2±1.2 % and 86.3±2.2%, respectively), indicating that the biostimulating effect of acetate only occurred during reactor startup, and that further addition resulted in a slight but significant inhibiting effect of acetate on TBBPA microbial transformation. Acetate is a key intermediate product in anaerobic digestion. This product is generated through the activity of acetogenic bacteria (Wang et al., 2013), which convert volatile fatty acids (VFAs) such as propionate, and butyrate, generated through microbial fermentation and acidogenesis, to acetate, which can further be utilized by acetoclastic methanogens to produce methane (Venkiteshwaran et al., 2015). Although none

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of the TBBPA-degrading strains isolated so far have been shown to use acetate instead of H2 as an electron donor, e.g. *Dehalobacter* sp. (Zhang et al., 2013), *Dehalococcoides mccartyi* (Yang et al., 2015), *Pseudomonas* sp. and *Streptococcus* sp. (Peng et al., 2014), other dehalogenating species possess this capability, e.g. *Desulfovibrio dechloracetivorans* (Sun et al., 2000), *Desulfuromonas* spp. (He et al., 2002; Krumholz, 1997; Sung et al., 2003), *Geobacter lovleyi* (Sung et al., 2006), and *Thrichlorobacter thiogenes* (De Wever et al., 2000). In addition, acetate has been previously reported to be a preferred electron donor over H2 for microbial dehalogenation in environmental samples (He et al., 2002; Lee et al., 2007). Therefore, it is possible that the dehalogenating microbial population present in our reactors might have been dominated by acetotrophic species over hydrogenotrophic species (He et al., 2002), hence explaining the stimulating effect of acetate addition on TBBPA microbial degradation. During startup however, it is likely that the syntrophic interactions between the key microbial groups involved in the digestion process were not yet fully established (Goux et al., 2016). Consequently, it is also likely that acetogenic bacteria were not yet generating acetate in our reactors. Therefore, the initial TBBPA microbial degradation observed within the first 17 days in the metabolic reactor might have only been supported by the limited amount of acetate initially present in the activated sludge used to seed our reactors. Thus, the interruption of TBBPA degradation observed in the metabolic reactor between Days 17 and 34 may be attributed to acetate depletion, and the restart of TBBPA microbial degradation at Day 34 could be explained by the fact that at this point, acetogenic microbial community had become functional, hence able to generate the acetate necessary to support TBBPA microbial degradation. Therefore, the effect of adding acetate after startup might have led to the accumulation of acetate in the reactor, which might have been the cause of the observed inhibition of TBBPA degradation. Indeed, accumulation of VFAs, such as acetate, has been shown to decrease the sludge pH, which can have inhibitory effects of anaerobic digestion processes (Franke-Whittle et al., 2014).

The following TBBPA spikes were degraded much faster than the initial TBBPA spike (Fig. 1). Unfortunately, as mentioned above, the time intervals at which sludge samples were collected in this study do not allow us to determine with high enough resolution how long it actually took for the microbial community to degrade each additional TBBPA spike. Indeed, the following spikes could have been degraded within a day or even within an hour following their addition. For the same reason, it is not possible to determine if there was a progressive increase in TBBPA degradation rates as a result of domestication of the microbial community to TBBPA exposure, as others have suggested (Li et al., 2016; Peng et al., 2017b). Indeed, it has been proposed that repeated exposure to TBBPA (i.e., domestication) could lead to a compositional shift towards microbial taxa with the capacity to degrade TBBPA, hence a gradual increase of TBBPA removal rate over time. In particular (Peng et al., 2017b) who tested the effect of domestication time on a sewage sludge microbial community observed a significant and gradual increase in TBBPA degradation rate with increasing domestication time. In their work, after 240 days of acclimation the domesticated microbial community was able to degrade 96% of the spiked TBBPA in 20 days, which was remarkably higher than after shorter domestication times. Although both studies used similar TBBPA concentrations (0.5 mg/L and 0.4 mg/L in this study), the TBBPA removal rate was substantially higher in the present study despite the shorter

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domestication time applied. For example, after 83 days of domestication time, the microbial community was able to degrade 96.2±0.5% and 98.4±0.3% of the fourth TBBPA spike in only 5 days at the most in the metabolic and co-metabolic reactor, respectively (Fig. 1). Such differences, however, can likely be attributed to differences in initial seeding material and composition of the associated microbial community, experimental setup, and reactor operating conditions.

3.2 Microbial community taxonomic composition

In order to investigate if repeated TBBPA exposure, or domestication of the microbial community, led to a shift towards a community dominated by a few taxa to whom TBBPA debromination could be attributed, a taxonomic characterization of the microbial community in TBBPA and control metabolic reactors was performed using 16S rDNA amplicon-based MiSeq Illumina sequencing. A total of 2,229,699 reads that clustered into 1,331 OTUs were obtained. Chao1 individual-based rarefaction curves (Fig. S2) and Good's coverage values (Table S1) indicate that the microbial communities were adequately sampled. Over 70% of the microbial community was represented by Proteobacteria, Bacteroidetes, Actinobacteria, and Chloroflexi, (Fig. S3). Although the 341F and 805R PCR primers used in this study were designed to target both bacterial and archaeal taxa (Takahashi et al., 2014), only six archaeal OTUs, that accounted for only 0.2% of the reads obtained, were detected (Fig S3). This number is much lower than previously reported on anaerobic sludge systems (i.e., ~6%; (Guo et al., 2015), and is likely the result of a preferential amplification of the primers towards bacterial taxa, as noted in our previous study (Lefèvre et al., 2018). Although the microbial communities received repeated addition of high concentrations of TBBPA, no particular taxon to which TBBPA degradation could be attributed became dominant in the TBBPA reactors over time as reported in previous studies (Li et al., 2016; Merlino et al., 2015; Peng et al., 2017b; Zhang et al., 2013). On the contrary, TBBPA and control reactors continuously shared more than 95% of their OTUs (Fig. S4), and the species richness and diversity in the TBBPA reactor at later sampling time points was not significantly different to that at Day 0 (Table S1). The weighted UNIFRAC matrix-based nMDS analysis (Fig. 2) clearly showed that samples clustered by time points and not by treatment, and the analysis of similarity (ANOSIM) confirmed that TBBPA addition was not the factor driving microbial community structure, which is consistent with our previous study (Lefevre et al., 2016). This indicates that the bulk of the microbial community was able to sustain the known toxic effects of TBBPA and BPA (Matsumura et al., 2015) without notably altering its taxonomic structure. This finding also suggests that the taxa responsible for the observed TBBPA degradation likely represent a small fraction of the microbial community. Nevertheless, an efficient response leading to TBBPA degradation was triggered. Thus, it appears that anaerobic sludge microbial communities present a high resistance to TBBPA exposure, which suggests that most microbial populations responsible for key processes in wastewater treatment would likely be able to carry on their function despite potential future increases in TBBPA concentration.

In an attempt to identify these TBBPA-degrading taxa, 'sensitive responders' to the presence of TBBPA were fetched out of our dataset. In accordance with Dai et al. (Dai et al., 2016), sensitive responders are defined as taxa (i.e., OTUs) whose relative abundance significantly

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increases, in the case of positive responders, or decreases, in the case of negative responders, by at least a factor of two in the TBBPA-supplemented reactor relative to the control reactor, at least at one time point over the course of the experiment. Therefore, taxa identified as positive responders would potentially be responsible for the observed TBBPA degradation, and taxa identified as negative responders could be taxa that are either unable to sustain TBBPA and BPA toxicity, or taxa that are outcompeted by TBBPA degraders. Among the 1,331 OTUs detected in this study, 93 positive and 77 negative responders, were identified (Fig. S5). In order to validate our 'sensitive responder' approach, additional nMDS and ANOSIM analyses only including responding taxa were performed (Fig. S6). These analyses showed a clear separation between the responder's communities collected from the TBBPA and the control reactor, especially after the second TBBPA spike. Although the results of the ANOSIM confirmed that TBBPA exposure had a significant effect on the responder's community, time was still the dominant factor driving the taxonomic composition dynamics of the responding community. Therefore, despite the fact that its taxonomic composition was not steady yet, the TBBPA degrading community was able to readily degrade TBBPA early on during reactor startup, indicating a degree of functional flexibility within the degrading community. Positive and negative responders represented overall 5.0, and 3.7% of the entire community relative abundance, respectively, and contrary to what was expected as a result of TBBPA repeated exposure, the overall relative abundance of the positive responders did not gradually increase over time (Fig. S7). This suggests that once steady conditions were established in the reactor, prolonged domestication time did not lead to a shift towards a community dominated by a few degrading taxa.

Members of the responding community were distributed across 25 phyla (Fig. 3A), and more than 50% of them (in terms of relative abundance) were affiliated to the dominant phyla Proteobacteria, Actinobacteria, and Chloroflexi (Fig. 3B). Although Bacteroidetes was also one of the most represented phyla in our reactors, responding bacteroidetes accounted only for 1.5% of the Bacteroidetes population (Fig. 3A), suggesting little involvement in TBBPA degradation. While Proteobacteria-affiliated responders were mostly negatively affected by the presence of TBBPA (Fig. 3B), most Actinomycetes-affiliated responders were positively affected (Fig. 3B), making them potential TBBPA degraders candidates, along with some Chloroflexi, Firmicutes, and Planktomycetes-related OTUs (Fig. 3B). Of all OTUs recovered in this study, only 22 related to the genera *Dechloromonas, Sulfurospirillum, Acetobacterium*, *Desulfovibrio*, and *Clostridium*, known to encompass species of dehalorespirers (Shukla et al., 2014; Tong et al., 2015), were detected. Among these 22 OTUs, only one, related to *Clostridium tyrobutyricum*, was identified as positive responder. Thus, adopting a more exploratory approach, such as the metagenomic approach used in this study, might be preferable over targeting for already known dehalogenators, especially when studying emerging halogenated compounds such as TBBPA. Among the 93 positive responders detected, 11 were qualified as positive responders in more than one sampling date (Fig. S8). These included the *Clostridium tyrobutyricum*-related OTU just mentioned, as well as another OTU related to the order of Clostridiales (Fig. S8). Only in the TBBPA reactor, the relative abundance of these two OTUs clearly increased overtime (Fig. S8), making these two taxa potential TBBPA degraders candidates. This is also supported by other studies that evidenced a link between the enrichment of Clostridiales-related

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phylotypes and the reductive dehalogenation of chlorinated and brominated compounds (Chen et al., 2010). Two other potential TBBPA degraders related to the environmental wastewater-sludge group Blvii28 also stood out. This group from which environmental sequences are commonly detected in anaerobic wastewater, does not contain cultivated representatives so far (Su et al., 2014), which limits our understanding of the ecophysiology and metabolism of its members. However, sequences affiliated to this group were also detected in our previous study in which a link between a Blvii28 taxon and TBBPA degradation was suggested (Lefèvre et al., 2018; Lefevre et al., 2016). More surprisingly, an OTU related to the obligate intracellular bacterium Chlamydia was part of the positive responders detected (Fig. S8). To the best of our knowledge, there is no previous evidence indicating the involvement of Chlamydia in halogenated compound degradation. However, the development of Chlamydia-related taxa in wastewater could be an unwanted consequence of TBBPA concentration increase. Indeed, although environmental Chlamydia are ubiquitous in wastewater sludge and live within free-living Amoeba and other protozoan hosts, they might also present a pathogenic potential for humans (Coulon et al., 2012). Thus, a possible development of Chlamydia species in wastewater sludge as a result of an increase of TBBPA concentration could pose the risk of their dissemination into water bodies that receive wastewater effluents, hence increase risks of human exposure (Collingro et al., 2005). Finally, four OTUs belonging to the Parcubacteria superphylum (i.e., OD1) of the Candidate Phyla Radiation were also qualified as positive responders to TBBPA. Species within the Parcubacteria appear to be obligate anaerobe and likely live in symbiosis with a wide range of other prokaryotic or eukaryotic members of the community (Nelson and Stegen, 2015). Particularly, one taxon was observed as an endoplasmic symbiont of a free-living Ciliophora (Gong et al., 2014), which can represent a dominant fraction of the eukaryotic microbial community in WWTPs (Chouari et al., 2017; Matsunaga et al., 2014). The detection of positive-responder OTUs related to protozoan-associated bacteria could indicate that symbiotic associations within wastewater systems might be much more prevalent than currently thought, and that the presence of TBBPA might stimulate the establishment of such symbiotic relationships within the community. A closer look at the eukaryotic microbial communities in WWTPs and their symbiotic associations with prokaryotic organisms might lead to a better understanding of the microbial processes driving wastewater treatment as well as the effect of perturbations on such processes. Therefore, unlike what was initially expected, repeated exposure to TBBPA did not result in the enrichment of a few taxa to which TBBPA degradation could be attributed. However, the high taxonomic resolution of the analysis performed in this study provides new indication regarding the taxa that might be involved in TBBPA degradation.

3.3 Methanogenic community dynamics

Although the direct involvement of methanogens in TBBPA degradation has been previously refuted, TBBPA microbial degradation has been shown to be stimulated under methanogenic conditions (Iasur-Kruh et al., 2010). In addition, archaeal communities have been previously shown to be involved in the production of biogas, a valuable by-product of the anaerobic digestion of wastewater sludge. Therefore, in order to explore the effect of TBBPA exposure on methanogenesis, the dynamics of archaeal populations were monitored at Days 0, 11, 24, 31, 41, 55, 70, 88, and 111 in the TBBPA and control metabolic reactors using qPCR (Fig.

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4). More specifically, qPCR assays targeting the whole archaeal and bacterial populations, as well as classes of acetoclastic (i.e., Methanosarcinales), and hydrogenotropic methanogens (i.e., Methanococcales, Methanobacteriales, and Methanomicrobiales) were used. First, the ratio archaeal:bacterial 16S rRNA gene copy number was never higher than 1% over the course of the experiment, which is in the range of previously reported values (Lefevre et al., 2016). However, it should be noted that the number of 16S rDNA copies has been shown to be lower in archaeal than in bacterial genomes (Stoddard et al., 2014). Thus, it is likely that the actual proportion of archaeal cells in our reactors might have been slightly higher than that indicated by this ratio. The archaeal 16S rDNA (ARC) qPCR assay, and the assay targeting the *mcrA* gene, which is only found in methanogenic archaea (Alvarado et al., 2014), revealed a similar temporal dynamic pattern (Fig. 4B, C). Given that most Archaea are likely to be methanogens in anaerobic digesters (Yu et al., 2015) this result was expected. Both ARC and *mcrA* assays revealed a progressive increase of the density of the overall methanogenic population over time, which is indicative of the establishment of methanogenic conditions in our reactors. However, while the ARC assay revealed an archaeal 16S rDNA copy number significantly higher in the control than in the spike reactor at day 70, the *mcrA* assay detected a significantly higher *mcrA* gene copy concentration in the TBBPA-spiked reactor than in the control at Days 88 and 111. These differences can be explained by the fact that the ARC and *mcrA* primer sets present taxonomic biases (Wilkins et al., 2015). Indeed 16S rDNA amplicon libraries usually show less taxonomic diversity and richness than *mcrA* amplicon based libraries (Alvarado et al., 2014). In addition, only one or two copies of the *mcr*A gene have been found in sequenced genomes of methanogenic taxa, as opposed to 1 to 4 copies for the 16S rRNA gene (Alvarado et al., 2014). Thus, the *mcrA* gene is generally considered to be more reliable and accurate to determine the dynamics of methanogenic populations (Ziganshin et al., 2016). Besides the *mcrA* gene has been shown to be an accurate biomarker of methane yield (Traversi et al., 2012). Therefore, based on the results of the *mcrA* assay, the fourth (at Day 83) and fifth (at Day 104) TBBPA spikes were followed by a significant increase of *mcrA* gene copy number, indicative of an increase of methane production in the TBBPA-spiked reactor (Fig. 4C), increase which might likely be related to the observed significant development of hydrogenotrophic methanomicrobiales in the TBBPA reactor at Days 88 and 111 (Fig. 4F).

The detection of both hydrogenotrophic Methanococcales, Methanobacteriales, and Methanomicrobiales, and acetoclastic Methanosarcinales (Fig. 4D–G) indicated that both acetoclastic and hydrogenotrophic methanogenesis pathways were occurring in our reactors. As it is usually the case in anaerobic digesters (Alvarado et al., 2014), the methanogenic population was dominated by members of the Methanomicrobiales, Methanobacteriales, and Methanosarcinales, whereas members of the Metanococcales were the least represented. At Days 0 and 11, hydrogenotrophic methanogens represented 60 to 80% of the methanogenic population. However, the low number of archaeal 16S rDNA and *mcrA* copies measured at these time points (Fig. 4B, C), likely corresponded to the archaeal community initially present but non-active during the startup phase of our reactors. Starting at Day 24 though, while methanogenic conditions were establishing in our reactors, as indicated by the increase of *mcrA* copies, the acetoclastic population became dominant, representing between 60 and 90% of the methanogenic population. Thus, acetoclastic methanogenesis

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was the dominant microbial pathway for methane production in our reactors, as usually observed in anaerobic digesters (Alvarado et al., 2014). This also indicates that once methanogenic conditions were established in the reactors, acetate was likely no longer a limited resource. Although the acetoclastic pathway prevailed in both reactors until the end of the experiment, at Day 41, when 67.1±3.1% (Fig. 1) of the first spike of TBBPA had been transformed, the proportion of hydrogenotrophic methanogens slightly increased in the TBBPA-spiked reactor relative to the control (Fig. 4H). The more TBBPA spikes the reactor received, the greater this increase became. Therefore, although acetoclastic methanogenesis was overall the prevailing pathway of methane production in our rectors, TBBPA addition and its degradation stimulated the activity of hydrogenotrophic methanogens. Methanomicrobiales probably largely contributed to this observed increased in hydrogenotrophic methanogenesis, as indicated by their significantly higher density in the TBBPA reactor after the fourth and fifth TBBPA spikes (Fig. 4F). This result was quite unexpected as it is generally considered that dehalogenators are in direct competition with hydrogenotrophic methanogens for the use of H2 as electron donor (David et al., 2015). However, in the present study, despite the fact that high dehalogenating activity was occurring, the proportion of hydrogenotrophic methanogens increased in the TBBPA-spiked reactors, again suggesting that microbial taxa responsible for TBBPA debromination in our reactors might have preferentially used acetate over H2 as electron donor for the reductive debromination of TBBPA.

4. Conclusions

Once steady conditions were established in the reactors, efficient microbial transformation of TBBPA to BPA took place. Surprisingly, repeated exposure to high concentrations of TBBPA did not result in a drastic compositional shift towards a community dominated by a few taxa to which TBBPA degradation could be attributed. In fact, the bulk of the microbial community did not ostensibly alter its taxonomic composition, and only a small fraction of the community responded to TBBPA. Responding taxa most potentially involved in TBBPA transformation affiliated to Clostridiales as well as members of the environmental wastewater sludge group Blvii28. TBBPA also triggered the development of potential symbionts of eukaryotes such as Chlamydia- and Parcubacteria-related taxa, suggesting the importance of studying the symbiotic relationships prevailing in WWTPs. Acetate only had a biostimulating effect during the startup phase of our reactors, when acetogenesis was likely not yet occurring, hence acetate was a limited substrate. This suggests that acetate could be primarily used as a direct electron donor, instead of H2, for the microbial degradation of TBBPA. In addition, the presence of TBBPA clearly resulted in an increase of hydrogenotrophic methanogens, indicating that TBBPA degraders were not in direct competition with hydrogenotrophic methanogen for H2, hence supporting the hypothesis that TBBPA degrading taxa might have been primarily using acetate as an electron donor for the degradation of TBBPA. The present study, by providing a comprehensive characterization of the microbial communities and processes related to TBBPA degradation hopes to give insights into how secondary wastewater treatment processes could be further improved to effectively remove TBBPA whose concentrations are expected to increase in the near future.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A small fraction of the microbial community responded to TBBPA exposure
- TBBPA-degrading taxa did not become dominant after domestication
- Acetate was likely used as electron donor for the reductive debromination of TBBPA
- Hydrogentrophic methanogens were stimulated in the presence of TBBPA

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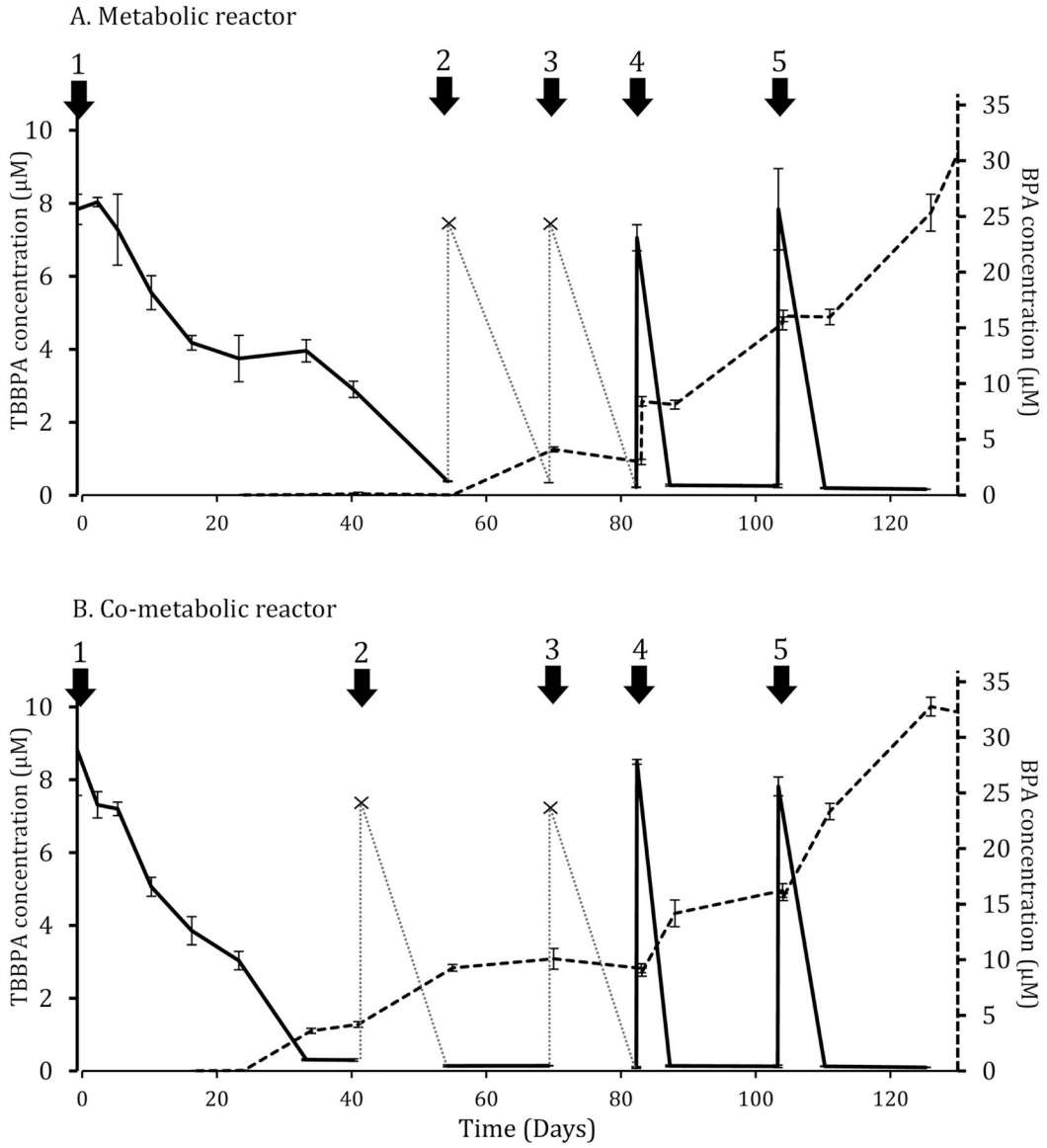

Figure 1.

Concentration of TBBPA and BPA in the sludge reactors. Concentration of TBBPA (solid line, left y-axis) and BPA (dashed line, right y-axis) in the metabolic (A) and co-metabolic (B) TBBPA reactors overtime. Error bars represent standard deviation from the mean. Numbered arrows represent the TBBPA spikes (target concentration of 7.3 μM). Cross marker symbols and dotted thin lines indicate that the concentrations of TBBPA for the second and third spikes were not actually measured in the reactors but were calculated based on the concentrations of the TBBPA stock solution used to spike the reactors.

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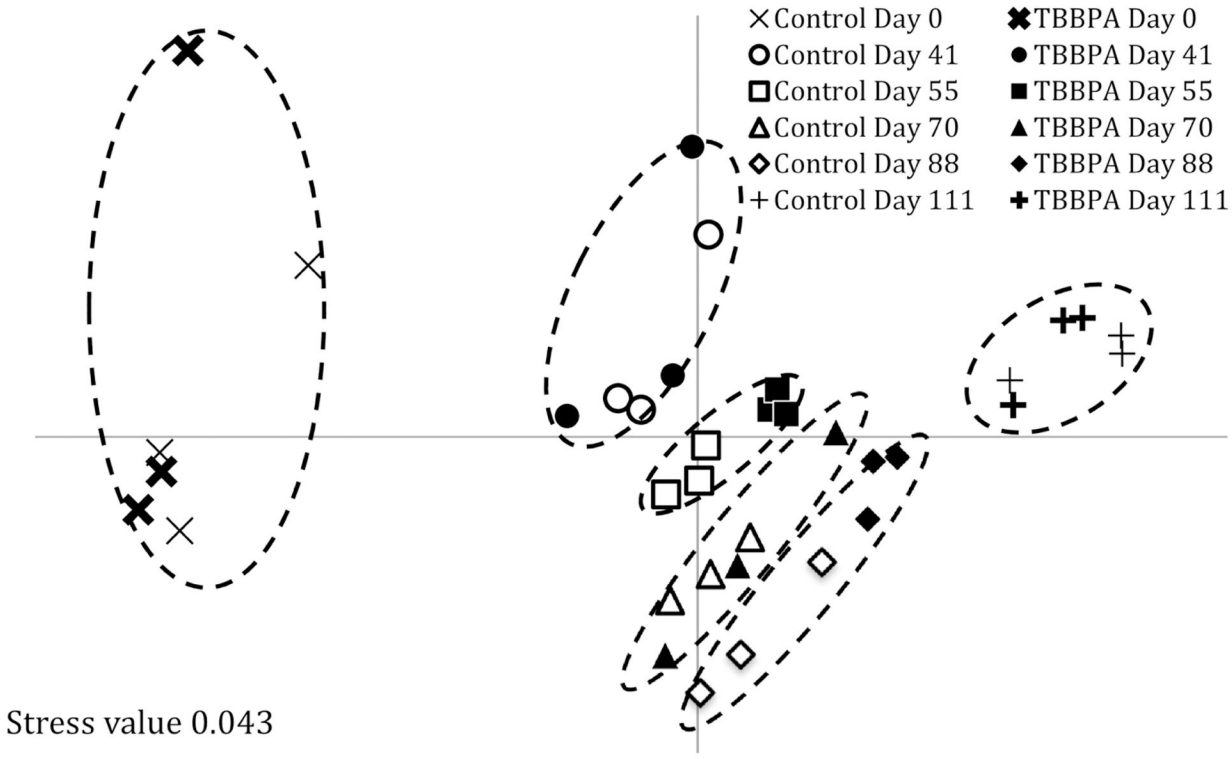

	Factor tested	
	Time	Treatment (TBBPA exposure)
R-value (p-value)	0.73 (0.001)	Failure to reject H_0

Figure 2.

Weighted UNIFRAC matrix-based non-metric multidimensional scaling (nMDS) plot, and analysis of similarity (ANOSIM) results of the whole microbial community. The calculated stress value is ≤ 0.05, which indicates an excellent representation of the samples in a two-dimensional nMDS plot. The table presents the results of the ANOSIM, with the null hypothesis (H_0) stating that the community composition does not differ between days or treatments. H0 is rejected if p≥0.05. The closer the R-value is to 1, the more difference there is between the groups tested in terms of community composition.

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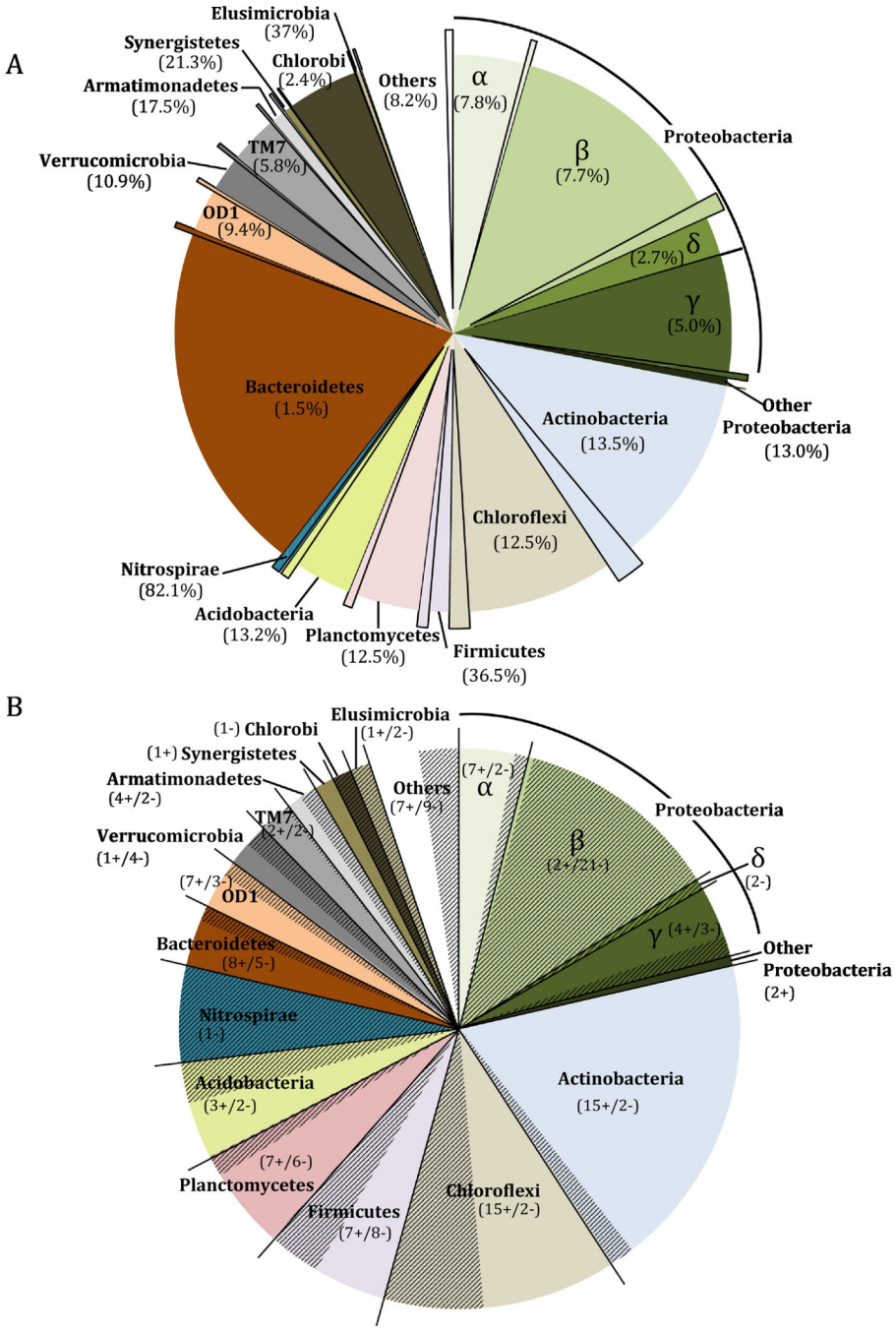

Figure 3.

Phylum-level distribution and relative abundance of the responding community. A: Pie chart representing the overall proportion of responders (pulled out slices; expressed in % of reads in parenthesis) within each major phylum (and sub-classes of the Proteobacteria). B: Pie chart representing the relative proportion (expressed in % of reads) of positive (plain color) and negative (hatched color) responders. In parenthesis are indicated the number of positive and negative OTUs. For both pie charts, ‘Others’ included Cyanobacteria, Chlamydiae, Archaea, Spirochaetes, OP11, WS3, WS6, NKB19, BRC1, and GNO4.

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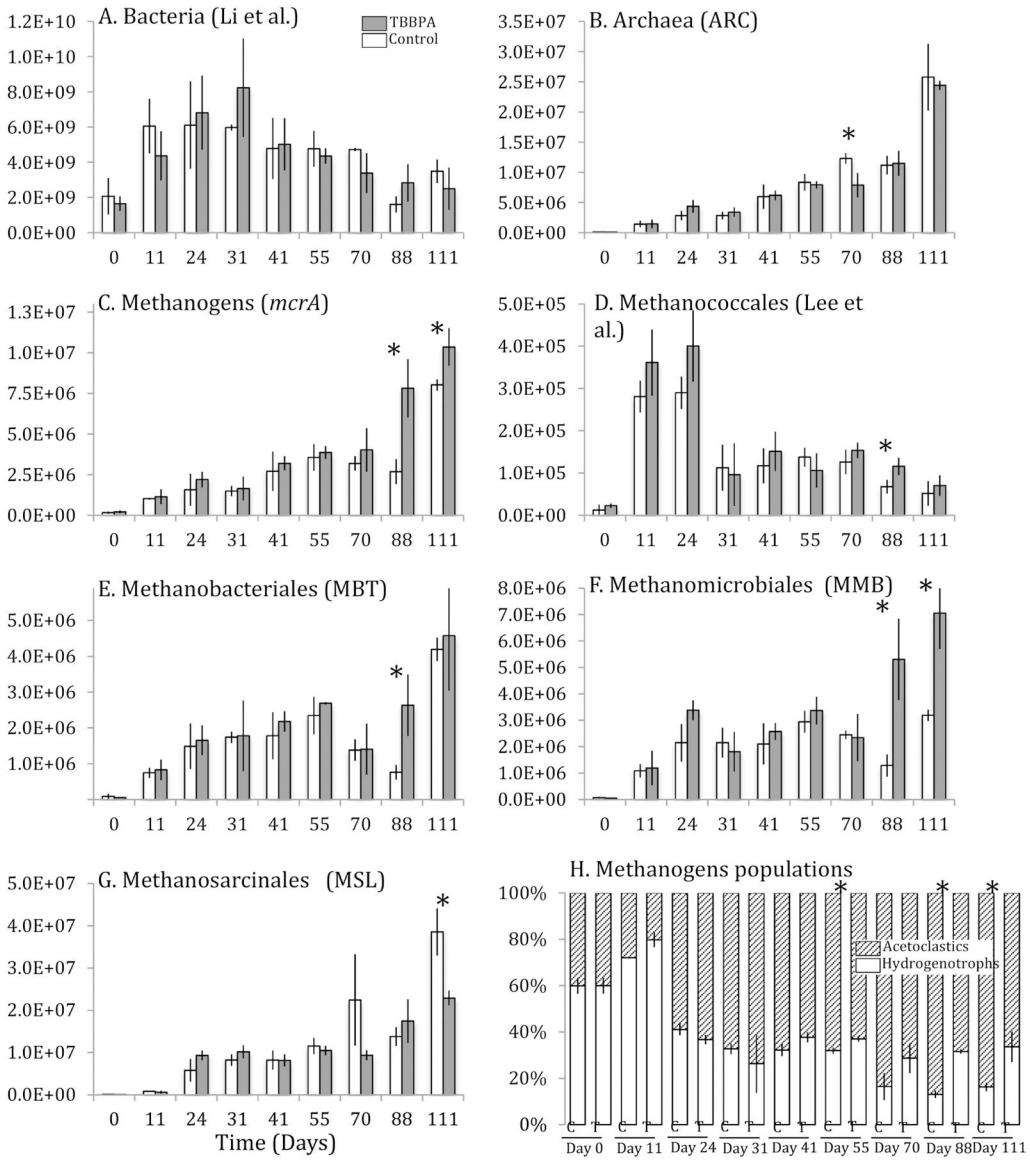

Figure 4.

Dynamics of methanogenic, archaeal, and bacterial populations. Microbial population dynamics of A) Bacteria, B) Archaea, C) methanogens, D) Methanococcales, E) Methanobacteriales, F) Methanomicrobiales, and G) Methanosarcinales in the control ('C') and TBBPA ('T') metabolic reactor in copies/mL of sludge, as measured by qPCR. The relative abundance of Acetoclastic (i.e., Methanosarcinales), and Hydrogenotropic methanogens (i.e., Methanococcales, Methanobacteriales, and Methanomicrobiales) is also represented (H). The Efficiency and R^2 calculated from the qPCR standard curves were between 86.7 and 97.2%, and 0.911 and 0.999, respectively. Error bars represent the standard deviation from the mean. Asterisks indicate significant differences between control and TBBPA reactors, according to a t-test (p= 0.05) performed for each sampling day.