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Tetrabromobisphenol A (TBBPA) degradation in anaerobic biosystems: from bioengineering to meta-omics

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WILLIAME VIEIRA MACÊDO

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Para a minha família: Mainha (Sonia), painho (Wellington), irmã (Gabriela) e cunhado (Jonath).
Para meu tio José Barbosa de Macêdo (Tio Nêm) (*in memoriam*): um dos 616.000 brasileiros vítimas do Covid-19.

To my family, mom (Sonia), dad (Wellington), sister (Gabriela), and brother-in-law (Jonath).
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(Abaixo, em português)

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Most importantly, I will teach them that *academia* is not a race and your job is not your life – is a part of it. We are not defined by our publications and H-index. We are not our curriculum. And finally, that we are not working towards publishable results - they are just a consequence and not the main goal: that's what I strongly believe. I am, once again, thankful for Marcelo. That was the message that we always got from him and I wish everyone would have had the same opportunity during their "PhD".

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Em português

Durante o meu doutorado, eu não poderia ter sido mais feliz e me sentido mais inspirada profissionalmente. Tive a sorte de trabalhar com um tema que me apaixonei, fui supervisionada por um ser humano e professor incrível, fiz parte de dois grupos que admiro. Nunca me vi parte das discussões sobre "a vida acadêmica" em que muitos se vêem em sofrimento, privação de sono, depressão, "não ter vida durante o doutorado", ou ter má supervisão. Nunca fui pressionada ou motivada a ultrapassar meus próprios limites. Eu fui feliz em cada experimento, fiquei realmente surpresa com o quanto que aprendi e absolutamente amei desempenhar o meu trabalho.

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“Some say our destiny is tied to the land, as much a part of us as we are of it. Others say fate is woven together like a cloth, so that one’s destiny intertwines with many others. It’s the one thing we search for, or fight to change. Some never find it, but there are some who are led.”

Brave, my mom’s and my favorite Disney movie.

“Dizem que nosso destino está ligado à nossa terra, que ela é parte de nós assim como nós somos dela. Outros dizem que o destino é costurado como um tecido, de modo que o destino de um se entrelaça ao de muitos outros. É a única coisa que buscamos, ou que lutamos para mudar. Alguns nunca encontram o destino, mas outros são levados a ele.”

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Resumo (em Português)

Macêdo, W. V. (2022). Degradação anaeróbia do micropoluente tetrabromobisfenol A (TBBPA): da engenharia à meta-ômica (Tese de doutorado). Escola de Engenharia de São Carlos, Universidade de São Paulo, São Paulo.

A presença de micropoluentes em ambientes aquáticos está geralmente relacionada ao lançamento de efluentes de estações de tratamento de esgoto doméstico, industrial e hospitalar em corpos hídricos. O Tetrabromobisfenol A (TBBPA) é um retardante de chama bromado aplicado em plásticos, revestimentos, materiais de construção, e produtos eletrônicos que apresenta risco potencial para a saúde humana e para o meio ambiente por ser um interferente endócrino, imunotóxico e neurotóxico. A otimização de sistemas anaeróbios para a degradação de micropoluentes e o microbioma envolvido neste processo permanece ainda pouco explorado. Nesta tese de doutorado, uma série de experimentos foram realizados para ampliar o conhecimento sobre algumas das lacunas relacionadas à biodegradação do TBBPA. Inicialmente, este trabalho propõe uma técnica de microextração líquido-líquido de alta precisão e sensibilidade, seguida por separação por cromatografia líquida de alto desempenho acoplada ao método de ionização por *electrospray* e detecção de espectrometria de massa em tandem (HPLC-ESI-MS/MS), para quantificar a presença de TBBPA em matrizes ambientais complexas. Em sequência, através da associação de uma série de experimentos de degradação e multiômica, fomos capazes de identificar os microrganismos que participam ativamente da degradação do TBBPA em concentrações ambientalmente relevantes e sua possível expressão funcional quando expostos a longo prazo ao poluente. Os resultados provenientes de quatro biorreatores anaeróbios sugerirem a fase específica da digestão anaeróbia em que a degradação de TBBPA ocorre, a relevância da adsorção do poluente na biomassa, a cinética de degradação e o perfil do microbioma ao longo do período operacional por sequenciamento do gene 16S rRNA. Além disso, a biomassa do biorreator de melhor desempenho foi aplicada em experimentos metaproteômicos (*protein stable isotope probing*, protein-SIP) e de metagenômica. Ao associar esses resultados, os microrganismos envolvidos na degradação do micropoluente foram identificados. A partir de genomas contendo genes que codificam os peptídeos marcados, proteomas foram gerados e as possíveis vias metabólicas de degradação do TBBPA foram descritas. Foram identificadas proteínas envolvidas na clivagem

hidrolítica das ligações carbono-halogênio, degradação do benzoato, transporte de compostos aromáticos e resistência a xenobióticos. Essas descobertas estão de acordo com os resultados iniciais baseados na correlação da eficiência de remoção do TBBPA e a caracterização temporal do microbioma, as rotas metabólicas de sistemas acidogênicos e à detecção de fenóis totais como um dos possíveis produtos de degradação.

Palavras-chave: Tetrabromobisfenol A; Biodegradção; Digestão anaeróbia; sequenciamento por amplicon; Metagenômica; Protein-SIP.

Abstract (In English)

Macêdo, W. V. (2022). *Tetrabromobisphenol A (TBBPA) degradation in anaerobic biosystems: from bioengineering to meta-omics* (D. Sc. thesis). Engineering School of São Carlos, University of São Paulo, São Paulo.

The presence of organic micropollutants (OMPs) in aquatic ecosystems is usually associated with the disposal of industrial and municipal effluents from wastewater treatment plants in water bodies. Tetrabromobisphenol A (TBBPA) is a brominated flame retardant applied to plastics, coatings, building materials, and electronics, and poses a serious threat to the human health and to aquatic ecosystems due to its endocrine disruptor, immunotoxic, and neurotoxic effects. The optimization of anaerobic biosystems for the degradation of micropollutants and the microbiome involved in the process remains little explored. In this doctoral thesis, a series of experiments have been conducted to extend the knowledge on some of the gaps related to the biodegradation of TBBPA. Firstly, an accurate and sensitive liquid–liquid microextraction technique followed by high performance liquid chromatography separation coupled with electrospray ionization tandem mass spectrometry detection (HPLC-ESI-MS/MS) method to determine the presence of TBBPA in complex environmental matrices is proposed. In sequence, by combining a series of degradation and multiomics experiments, we were able to identify the microorganisms actively degrading tetrabromobisphenol A (TBBPA) at environmentally relevant concentrations in anaerobic settings and their putative functional expression in long-term exposure to the pollutant. The results from a total of four anaerobic continuous bioreactors suggested the specific stage of the anaerobic digestion in which the degradation of TBBPA takes place, the relevance of the adsorption of the pollutant onto the biomass, the degradation kinetics, and the microbiome profile throughout the operational period by amplicon sequencing of the 16SrRNA gene. Additionally, the biomass from the best-performance bioreactor was applied in labelled metaproteomics (protein stable isotope probing, protein-SIP) and metagenomics experiments. By linking metagenomic, predicted functional, and metaproteomic data, the microorganisms involved in the degradation of the micropollutant were identified. From metagenome-assembled genomes (MAGs) containing coding sequences for the labelled peptides, predicted proteomes were generated and the putative metabolic pathways were described. Proteins involved in the hydrolytic cleavage of carbon-halogen bonds,

benzoate degradation, transport of aromatic compounds, and resistance to xenobiotics were identified. These findings are in agreement with the initial predictions based on the correlation of the bioreactor's performance and the temporal characterization of the microbiome, the metabolic routes undergoing acidogenic biosystems, and the detection of total phenols as one of the possible degradation products.

Keywords: Tetrabromobisphenol A; Biodegradation; Anaerobic digestion; Amplicon sequencing; Metagenomics; Protein-SIP

List of Figures

Lista of Tables

Table of Contents

General Introduction

Organic micropollutants (OMPs) are chemical substances that occur at extremely low concentrations (µg – ng·L^{-1} range) in aquatic environments. These chemicals threaten both human health and the aquatic ecosystems due to its adverse ecotoxicological effects, its recalcitrant properties, and ability to bioaccumulate in the food chain. Tetrabromobisphenol A (TBBPA) is the most used brominated flame retardant in the world and its biodegradation in wastewater treatment plants (WWTPs) remains little explored. Anaerobic bioreactors are one of the main technologies applied for the biotreatment of effluents in municipal and industrial WWTPs, however, these technologies are still inefficient to remove the more recalcitrant OMPs. Knowledge on the microbial communities performing natural attenuation of contaminated environments is crucial for optimizing biotreatment technologies and, even though the biodegradation of TBBPA has been investigated in the past 20 years, the knowledge on its bioconversion is still far from being transferable to wastewater treatment systems. The anaerobic digestion (AD) of TBBPA and the organisms acting on its degradation remains unclear.

The overall objective of this doctoral thesis was to extend the knowledge on the degradation of TBBPA in anaerobic bioreactors from bioengineering aspects to meta-omics. For this purpose, different approaches and techniques were applied throughout several experiments set-ups. These experimental set-ups were based on scientific hypothesis that yielded results fully published Research and Review Papers in high impact peer-reviewed journals and presented in this manuscript as chapters (*Chapters 1, 2, 3, 4, and 5*).

The general hypothesis of this thesis and the sub-hypothesis that were tested are presented below, as well as the specific goals.

General hypothesis: The anaerobic biodegradation of the flame retardant TBBPA at environmentally relevant concentrations (in the µg·L^{-1}) can be revealed by combining bioengineering and meta-omic studies.

Sub-hypothesis 1 (Chapter 2): Miniaturized liquid-liquid dispersive extraction can be an efficient method for quantifying TBBPA in complex environmental matrices and to measure its biomagnification potential in aquatic test-organisms.

Specific goals: To propose a simple and cost-effective analytical method to quantify TBBPA in environmental matrices and its biomagnification potential; To evaluate the applicability of the proposed method when quantifying TBBPA in domestic sewage, anaerobic sludge, and representatives of aquatic organisms (*Daphnia magna* and *Chironomus sancticaroli*).

Sub-hypothesis 2 (Chapter 3): TBBPA cometabolism occurs in a specific stage of the anaerobic digestion (AD).

Specific goals: To narrow down in which stage of the AD the degradation of TBBPA occurs at environmentally relevant concentrations (in the range of $\mu g \cdot L^{-1}$); To evaluate the degradation kinetics of TBBPA in an acidogenic and in a multi-stage methanogenic continuous bioreactor; To quantify TBBPA adsorption onto the biomass in continuous systems.

Sub-hypothesis 3 (Chapter 4): The hydrolytic activity in AD systems would benefit TBBPA bioconversion and the putative TBPPA-degraders could be identified by temporal biomass sampling and amplicon sequencing.

Specific goals: To study the performance of strictly acidogenic and a hydrolytic-acidogenic reactors on TBBPA biodegradation; To uncover the bacterial populations potentially involved in the pollutant breakdown by amplicon sequencing of the 16S rRNA gene of the biomass sampled throughout the operational period.

Sub-hypothesis 4 (Chapter 5): In-depth knowledge on the metabolic pathways and the identity of the organisms actively degrading TBBPA at environmentally relevant concentrations can be achieved by using a combination of metagenomics and labelled metaproteomics (protein stable isotope probin, protein-SIP) approach.

Specific goal: To identify the carbon fluxes and the functional groups of active TBBPA-degraders within the biomass supplied with ^{13}C–TBBPA in time-series sampling (50%, 75%, and 99.9% of TBBPA degradation efficiency); To suggest metabolic pathways of degradation based on the predicted proteome generated from metagenome-assembled genomes of the TBBPA degraders.

The presented doctoral thesis has been funded by the São Paulo Research Foundation (FAPESP) and conducted primarily at the University of São Paulo (years 2019 and 2020) and in collaboration with Aalborg University (year 2021). Figure 1 presents the main gaps addressed in each chapter of this thesis as a guide to the reader.

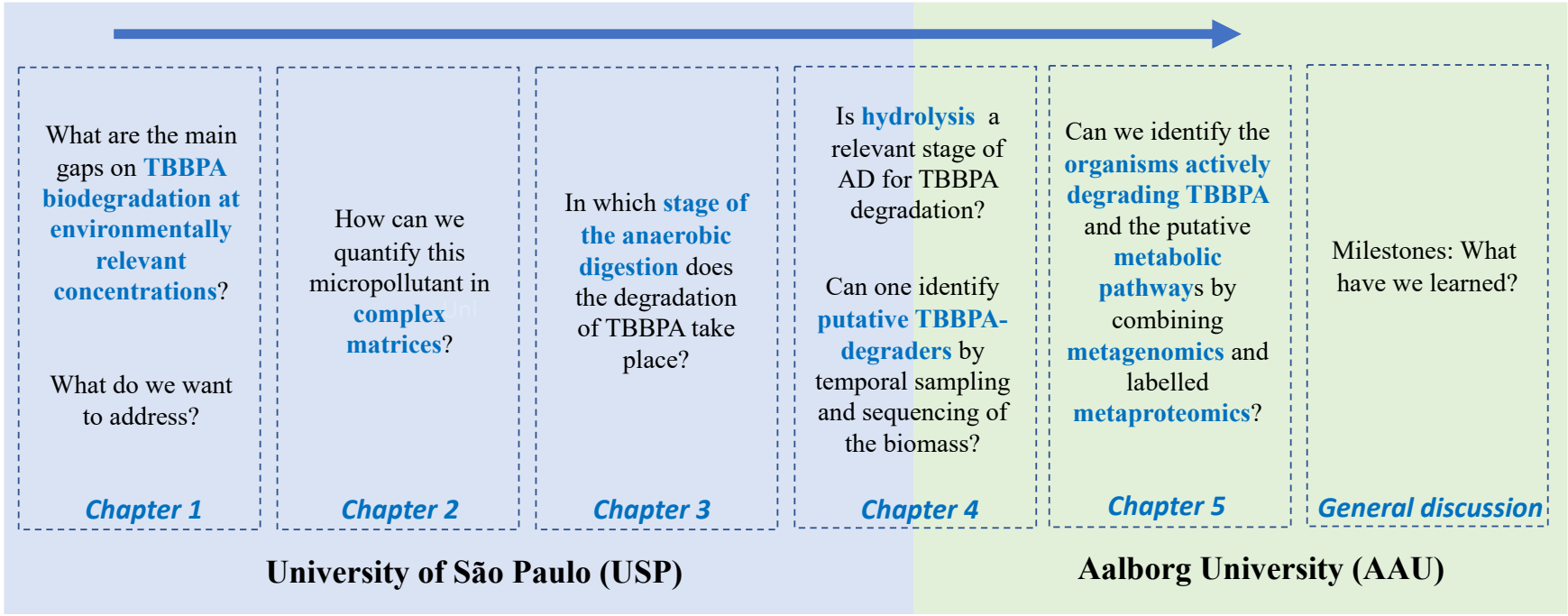

Figure 1 – Structure of this thesis and the main questions addressed in each chapter.

Rights to include published Review and Research Papers in my thesis

The chapters presented in this doctoral thesis have been fully published by **Elsevier**, **Springer**, and **Wiley** in high impact journals. All three publishers stated *that authors can include their articles in full or in part in a thesis or dissertation for non-commercial purposes without written permission, but subject to proper acknowledgment. This right extends to the posting of the thesis to the university's repository provided that if you include the published journal article, it is embedded in the thesis and not separately downloadable.*

List of Review and Research papers related to this doctoral thesis

Macêdo, W. V., Sánchez, F. E., & Zaiat, M. (2021). What drives Tetrabromobisphenol A degradation in biotreatment systems? 20(3), 729–750. https://doi.org/10.1007/s11157-021-09579-9 *(Presented in this manuscript as **Chapter 1**);*

Macêdo, W. V., Bernegossi, A. C., Sabatini, C. A., Corbi, J. J., & Zaiat, M. (2020). Application of Dispersive Liquid–Liquid Microextraction Followed by High-Performance Liquid Chromatography/Tandem Mass Spectrometry Analysis to Determine Tetrabromobisphenol A in Complex Matrices. ***Environmental Toxicology and Chemistry***, 39(11), 2147–2157. https://doi.org/10.1002/etc.4837 (*Presented in this manuscript as **Chapter 2***);

Macêdo, W. V., Duarte Oliveira, G. H., & Zaiat, M. (2021). Tetrabromobisphenol A (TBBPA) anaerobic biodegradation occurs during acidogenesis. ***Chemosphere***, 282, 130995. https://doi.org/10.1016/j.chemosphere.2021.130995. (*Presented in this manuscript as **Chapter 3***);

Macêdo, W. V., Poulsen, J. S., Oliveira, G. H. D., Nielsen, J. L., Zaiat, M. (2022) Tetrabromobisphenol A (TBBPA) biodegradation in acidogenic systems: One step further on where and who. ***Science of The Total Environment***, 808, 152016. https://doi.org/10.1016/j.scitotenv.2021.152016 (*Presented in this manuscript as **Chapter 4***);

Macêdo, W. V., Poulsen, J. S., Zaiat, M., & Nielsen, J. L. (2022). Proteogenomics identification of TBBPA degraders in anaerobic bioreactor. ***Environmental Pollution***, 119786. https://doi.org/10.1016/j.envpol.2022.119786 (*Presented in this manuscript as* ***Chapter 5***);

Macêdo, W. V.; Poulsen J. S.; Zaiat, M.; Nielsen, J. L. Genomic, proteomic, and metabolites characterization of TBBPA-degraders (*Enterobacter cloacae* strains). ***In prep and expected to be submitted by March of 2022.*** (*This research paper has not been included in this thesis*).

Chapter 1

What drives Tetrabromobisphenol A degradation in biotreatment systems?

Abstract: The growing concern on the fate of toxic and persistent micropollutants in aquatic ecosystems led to the need to comprehend how these substances can be converted into less harmful chemicals. Tetrabromobisphenol A (TBBPA) is the most used brominated flame retardant (BFR) worldwide and is often detected in water bodies and wastewaters. In the past 20 years, the degradation of TBBPA has been investigated in bioreactors with different microbial communities and under a variety of operational parameters. Comprehending TBBPA biodegradation contributes to a better understanding of other flame retardants environmental fate and the optimization of wastewater treatment biotechnologies. Even though many process-optimization procedures and investigations on mixed and isolated strain functions have been conducted, understanding metabolic processes on this micropollutant breakdown remains uncovered, especially in environmental settings. Different findings on how to optimize TBBPA bioconversion and the factors influencing cometabolic and metabolic reactions may mislead further studies or suggest arguable directions. For this reason, this review summarizes and critically discusses how the main environmental and operational parameters affect TBBPA biodegradation, the main degradation pathways and subproducts, and the microorganisms and enzymatic activities involved in this process, raising some questions that should be addressed in more in-depth studies.

Keywords: biodegradation; metabolism; biosystems; micropollutants.

Introduction

The micropollutant Tetrabromobisphenol A (TBBPA) is the most common flame retardant applied to electric and electronic equipment, epoxy resins, and plastic products to meet fire safety requirements and has been detected in air, dust, sediment, biota, and water. In water bodies, its concentrations range from few picograms per liter to 4.87 µg L^{-1} (Yang et al., 2012; Ni and Zeng, 2013; Wang et al., 2014; Zhou et al., 2014; Qu et al., 2016; Daso et al., 2017; Rothenbacher and Pecquet, 2018) and in sewage sludge samples, from undetectable to 1329 ng g^{-1}dw (dried weight, dw) (Gorga et al., 2013). The influent concentration of TBBPA in wastewater treatment plants

(WWTP) might be even higher due to its large production and usage, however, the tendency of TBBPA to be partially dibrominated into mono-, di-, and tri-BBPA contributes to a misrepresentation of its occurrence in wastewaters (Xu et al., 2021).

Concerns on TBBPA environmental occurrence have led to studies on technologies capable of converting this chemical into less toxic and less persistent compounds, considering that TBBPA is immunotoxic and neurotoxic, and may cause endocrine disruptions in organisms, associated with the development of cancer in the uterus of rats, maternal transmission between fish generations, and genetic mutation in frogs (Veldhoen et al., 2006; Covaci et al., 2009; Nyholm et al., 2008; Yang et al., 2012; NTP, 2014).

The biological degradation of halogenated micropollutants is assumed to be possible under all redox conditions by a variety of bacterial and archeal species (Hardman, 1991; Peijnenburg et al., 1992; Haggblom, 1992; Fetzner, 1998; Ghattas et al., 2017). In bioreactors, TBBPA degradation by microbial species has been investigated over the past 20 years, aiming at optimizing bioprocesses mostly by evaluating operational parameters (pH, temperature, concentration, carbon source, and other amendments) and understanding isolated bacterial strain functions (Ronen and Abeliovich, 2000; Iasur-Kruh et al., 2010; Chang et al., 2012; Peng et al., 2013; Peng et al., 2014; Yang et al., 2016; Fan et al., 2017; Islam et al., 2018; Cheng et al., 2019; Lefevre et al., 2019; Lin et al., 2020). Nearly all studies on TBBPA bioconversion have been conducted at high concentrations, compared to its environmental occurrence (in ng L^{-1} or μg L^{-1}), and by pure or enriched cultures under laboratory settings, which are too far from environmentally relevant conditions, but provide crucial insights into the metabolic mechanisms of TBBPA bioconversion. More recent research (Fan et al., 2017; Fan et al., 2020; Chen et al., 2019) has studied TBBPA degradation by mixed microbial cultures in concentrations closer to its occurrence in natural systems. However, these studies do not consider real wastewater composition and are still exploratory.

Information on what promotes this micropollutant degradation relies mostly on process efficiencies based on cause-effect responses: efficiency, removal rate, and half-life, in response to variations on pH values, carbon sources, temperature, bioaugmented microbiota, and other amendments. Understanding biochemical pathways, the transformation of degradation products, and the microorganisms and enzyme pool responsible for biocatalytic reactions in TBBPA degradation is still unknown and underexplored.

There have been no reviews so far that put together data of TBBPA biodegradation emphasizing bacterial metabolism. This review provides a critical overview on reports regarding TBBPA degradation and biotransformation by microorganisms, focusing on anaerobic and aerobic bacterial studies, by discussing the metabolic and enzymatic processes underlying TBBPA conversion. The aim of this paper is to outline and summarize knowledge on TBBPA bioconversion mechanisms in a critical way by elucidating relevant biochemical and operational aspects, evaluating its transferability to natural ecosystems, and suggesting further directions on the topic based on what is already known. Considering this objective, the following will be discussed: 1) the main environmental and operational parameters in bioreactors and how they affect TBBPA biodegradation, 2) an overview on TBBPA degradation in bioreactors under anaerobic and aerobic conditions, 3) the main degradation pathways and subproducts, and finally 4) the microorganism and enzymatic activities involved in this micropollutant biodegradation.

Environmental and operational parameters for TBBPA degradation

In bioreactors, the major reactions on the degradation of organic micropollutants (OMP) (hydrolysis, oxidation, and reduction) depend on many environmental factors such as redox potential, temperature, microbial community, pH, organic carbon content, and the physical and chemical properties of the OMP itself (Hardman, 1991; Peijnenburg et al., 1992; Haggblom, 1992; Fetzner, 1998). Before discussing the metabolic mechanism by which TBBPA is converted into byproducts, some environmental factors especially relevant for the fate of this micropollutant must be considered. The TBBPA concentration, its sorption capacity, and the effect of the pH on TBBPA chemical speciation and microbial activity will be further discussed because these parameters are intrinsically related to each other, have major effects on bacterial metabolism, and are often overlooked.

The effect of TBBPA concentration

TBBPA biodegradation in anaerobic, aerobic, and/or anoxic engineered biosystems, has been studied under concentrations that are much greater than its environmental occurrence (ng L^{-1} to μ L^{-1}). This fact may induce adaptations and microbial growth of specific strains, leading to metabolic routes that would not occur otherwise. As a consequence, conclusions towards

transformation products, co-metabolic preferences, microbial efficiency, and gene expression may be untrue in natural environments (Ghattas et al., 2017). Studies with a more realistic approach determine whether or not predicted metabolic pathways may be transferable from laboratory to environmental settings (Ghattas et al., 2017). TBBPA occurrence in the aquatic ecosystems usually does not support its use as a sole or main carbon source for microbial growth and biotransformation occurs through co-metabolism, in which its degradation is significantly accelerated by adding an exogenous carbon source (Brenner et al., 2006; Wei et al., 2018; Fernandez-Fontaina et al., 2016; Gonzalez-Gil et al., 2018).

TBBPA at concentrations in the order of mg L^{-1} or mg kg^{-1} may cause intracellular metabolism disruption and has been negatively correlated to biomass growth, besides affecting microbial ecology (the microbiome richness and diversity) (Xie et al., 2018; Wan et al., 2019). These effects are often overseen and underestimated when the biodegradation of TBBPA is represented by specific strains enriched over time or isolated from contaminated sites and that have known gene expressions related to dehalogenation, aromatic rings cleavage, and O-methylation processes, for example, being able to biotransform TBBPA at high concentrations and controlled conditions (An et al., 2011; Peng et al., 2013 b; Peng et al., 2014; Zu et al., 2014; Gu et al., 2016; Ma et al., 2017; Xu et al., 2018; Gu et al., 2018; Liang et al., 2019 a, 2019 b).

Additionally, TBBPA degradation may be positively or negatively associated with other substances present in wastewater, contaminated soils, and natural environments, such as anions (NO_3^-, HCO_3^-, SO_4^{2-}) and metals (Cu^{2+}, Fe^{II}, Fe^{III}, and Cd^{2+}) (Arbeli et al., 2006; Ma et al., 2017; Xu et al., 2018; Wang et al., 2019; Lin et al., 2020), besides other OMPs. Carbon sources are also involved in TBBPA removal by other means rather than co-metabolic or metabolic functioning of microbial communities: complexation to organic matter. TBBPA glycoside and TBBPA glucoside (subcategory of glycosides) is formed by fungi (Chen et al., 2019 b), microalgae species (Peng et al., 2014 b), and pumpkin plants (Hou et al., 2019) by glycosylation reactions in which the glycosyl substitutions of the metabolites were bonded with an oxygen atom of the phenolic hydroxyl groups of TBBPA through the O-1-glycosidic bond. These enzyme-mediated reactions contribute to TBBPA biotransformation and removal, even though they do not cause its degradation.

Knowledge on the biodegradation capacity of specific strains is extremely important to elucidate the mechanisms that may lead to TBBPA degradation, but it would hardly represent the degradation performance in Wastewater Treatment Plants (WWTP), contaminated soil, and aquatic

ecosystems. Furthermore, microbial communities from different environments and with distinct taxonomic compositions may perform similarly on micropollutant degradation with respect to the enzymatic transformation (Fenner et al., 2020), which may not be predicted by studies with isolated and acclimated strains neither at unrealistic concentrations.

The lack of information on TBBPA biodegradation by indigenous microbial communities in a complex medium at environmentally relevant concentrations may be related to the relatively new concern regarding micropollutant contamination. However, with no further studies, it would be impossible to predict and understand how its degradation could be optimized in WWTP and contaminated ecosystems.

pH

Another parameter that directly influences TBBPA removal from the medium is the pH value. The experimentally determined water solubility of TBBPA is 0.148, 1.26, and 2.34 mg L^{-1} at pH values of 5, 7, and 9, respectively at 21-25 ºC (Bureau, 2006), which is rarely in the range of concentrations studied in biodegradation tests (See Table 1.1). It has been extensively discussed and proved how pH affects bacterial and enzymatic activities (Singleton, 1994; Peng et al., 2013b; Peng et al., 2014), but what is often overlooked is the influence of pH on TBBPA chemical properties, as this parameter drives TBBPA speciation (modifying its hydrophobicity) and plays an important role in the partitioning to organic carbon and adsorption surfaces (Potvin et al., 2012; Zhang et al., 2013).

TBBPA has ionizable functional groups (with two acidic hydrogen atoms) that are affected by the medium pH, leading to chemical interactions that can affect both its transport and bioavailability in the environment and engineered systems. The values for pK_{a1} and pK_{a2} are 7.5 and 8.5, respectively, meaning that the ionized forms of TBBPA will become prevalent at pH from 7 to 8. At pH values lower than 7, TBBPA will be present essentially as the undissociated form, and therefore, it will be less soluble and likely to adsorb on surfaces at acidic medium ($pH < 7$) (Bureau, 2006). The strength of these chemical interactions is related to TBBPA chemical structure, bulk organic matter, and sorption area properties, including hydrophobicity, functional group content, and solution chemistry (Neale et al., 2009).

The optimal biotransformation efficiencies of TBBPA by some isolated strains (*Ochrobactrum* sp., *Comamonas* sp., *Pseudomonas* sp., and *Streptococcus* sp.) occurs at pH 7.0,

meaning that partial acid conditions would accelerate TBBPA degradation (An et al., 2011; Peng et al., 2013 b; Peng et al., 2014). Even though neutral and mildly acidic pH might be ideal for bacterial functioning, TBBPA removal might have also been driven by its speciation in the wide pH range studied. Lower pH values lead to greater adsorption capacity, and as a consequence, greater TBBPA removal, which was not explicitly accounted for by the authors. Moreover, An et al. (2011) reported that by testing optimal conditions for TBBPA degradation by *Ochrobactrum* sp. strain, the initial TBBPA concentration and pH had more significant effects on degradation efficiency than those of temperature and inoculum size, and the optimal pH for debromination activity differed from the optimal pH for cell growth. pH and TBBPA concentration are directly related to each other and to its bioavailability in different chemical species. When the pH effect is attributed solely to enzymatic activity, the relevance of TBBPA chemical speciation and bioavailability in the degradation mechanism is neglected.

Sorption

The sorption phenomenon is often overseen in studies regarding TBBPA and other flame retardants degradation and may interfere with false positives or negatives in predicted correlations of biological and biochemical results, super-estimating systems efficiency and mistaking degradation by removal, as removal of TBBPA from typical WWTP is likely due to a combination of sorption and biotransformation (Potvin et al., 2012; Islam et al., 2014; 2018). In a study conducted at a WWTP in China (Wang et al., 2020), sorption via biosolid was the major removal mechanism for different brominated flame retardants, with the pollutants' mass fraction in the biosolid as high as 91%. Hydrophobic interactions of a compound with the lipophilic cell membrane of microorganisms, which is influenced by the composition and pH of the sludge and the medium, organic matter content, biosurface, and environmental conditions (Ternes et al., 2004; Islam et al., 2016) may remove relevant amounts of pollutant from the liquid phase. Not only for TBBPA, but in general, the concentrations of micropollutants adsorbed in the solid phase of biological sludges (digested or aerobic) have been rarely measured (Petrie et al., 2015; Gonzalez-Gil et al., 2018). TBBPA quantification in both liquid and solid phases allow the differentiation of degradation from removal.

Sorption of micropollutants on sludge from WWTPs can be predicted based on the solid–water distribution coefficients (k_d) of the compound (Ternes et al., 2004). However, undergoing

biodegradation, dissolved and bounded TBBPA in a biological system, in a laboratory or in a WWTP, are not necessarily at equilibrium (Potvin et al., 2012). Furthermore, as previously mentioned, the uptake of TBBPA on solid particles decreases with increasing pH values as a function of TBBPA speciation distribution, and is affected by the presence of major anions often present in biological systems as NO_3^-, HCO_3^-, SO_4^{2-}, and HPO_4^{2-} (Zhang et al., 2013), which may vary in concentration throughout the bioreactor operation.

The dissolved fraction of OMPs has recently been considered as the only available and biodegradable fraction (Pomiès et al., 2013), but biotransformation may occur in both phases (soluble and sorbed) (Xue et al., 2010; Gonzalez-Gil et al., 2018). In fact, Gonzalez-Gil et al. (2018) attributed the greater biotransformation efficiency of fluoxetine, carbamazepine, and musk fragrances to their hydrophobicity, among the 28 chemicals investigated in the same study. The authors associated the substances' sorption on the biomass with an increase in the bioavailability and found no link between the partition coefficient (k_d) and the overall biotransformation efficiency. A studied conducted by McAvoy et al. (2016) supported this hypothesis for TBBPA, specifically. The authors investigated the biotransformation of TBBPA in digested sludge, soil, and sediments from a freshwater environment with radiolabeled ^{14}C-TBBPA.

By monitoring $^{14}CO_2$ and $^{14}CH_4$, only 1% of TBBPA was mineralized over 120 days, and despite the fact that most of the ^{14}C-TBBPA was immediately adsorbed on the sludge solids (the water layers contained less than 10% of the total initial concentration at all sampling intervals), the amount of ^{14}C-TBBPA in the solid extracts decreased from 95% on day 0 to 13% by day 56 and 2% by day 120. These extracts also contained three unknown biotransformation products that were ultimately transformed into BPA, confirming TBBPA biodegradation after being sorbed on solid particles.

Many studies (Peng et al., 2013; Fan et al., 2017; Fan et al., 2020; Chen et al., 2019) correlate a decrease in TBBPA degradation efficiency with increasing affluent concentrations justified by insufficient electron donors, biomass inhibition, or biomass inability, which is reasonable. However, sorption considerations of the micropollutants on the biomass and organic compounds, such as humic acid, known to form HA-bound residues and alter the fate of TBBPA (Zhang et al., 2013; Ma et al., 2017; Fan et al., 2020), are not taken into account, leading to arguable results.

An overview on the use of anaerobic and aerobic reactors to degrade TBBPA

Anaerobic reactors

To the best of our knowledge, the first study on TBBPA biotransformation was published 20 years ago. Ronen and Abeliovich (2000) studied the degradation of TBBPA, TBP, and BPA under anaerobic-aerobic conditions with contaminated river sediments as inoculum, and even though organic matter was used to compose the medium (tryptone, peptone, glucose, and yeast extract), the authors did not mention co-metabolic purposes. Their work firstly suggested that in anaerobic environments, TBBPA would undergo reductive dehalogenation and accumulate BPA, which would be further degraded in aerobic medium. Many other studies confirmed BPA accumulation (Vooderecker et al., 2002; Arbeli and Ronen, 2003; Arbeli et al., 2006; Iasur-Kruh et al., 2010; Liu et al., 2013; McAvoy et al., 2016; Lefevre et al., 2016; 2019; Wei et al., 2018; Jiang et al., 2020) and no further degradation under anaerobic conditions. In a later study, the same group related that TBBPA debromination is stimulated by organic compounds in co-metabolic reactions. (Arbeli and Ronen, 2003).

Voorderecker et al. (2002) established both methanogenic and sulfate-reducing conditions in anoxic estuarine sediments for TBBPA degradation. Even though their results suggested complete TBBPA dehalogenation to BPA with no further degradation in both conditions, in the sulfate-reducing condition dehalogenation was much slower, even though the lag phase was not related to the depletion of sulfate, which could be understood as a preferable electron acceptor. Instead, it was hypothesized that in the presence of ongoing sulfate reduction, lower levels of hydrogen or other sources of reducing equivalents prevailed more than in the methanogenic enrichments. Therefore, lower concentrations of electron donor may have resulted in the slower observed rates of dehalogenation (Vooderecker et al., 2002).

After Ronen and Abeliovich (2000), Arbeli and Ronen (2003), and Arbeli et al. (2006) findings, Iasur-Kruh et al. (2010) (same research group) conducted further studies on TBBPA degradation that corroborate with the hypothesis stated by Vooderecker et al. (2002) on the role of intermediates generated in the methanogenic process. The authors observed that when Archaeal species were chemically inhibited (by 2-bromoethanesulfonate, BES), TBBPA debromination was delayed and when bacterial species were inhibited (by ampicillin), the degradation did not occur at all. Afterwards, the effect of different electron donors (ethanol, lactate, acetate, and butyrate) suggested

that best results on TBBPA conversion were associated with carbon sources yielding H_2 production (ethanol>lactate>butyrate), while no conversion was observed when acetate was used, pioneeringly suggesting that debromination as a respiratory process prevails on anaerobic TBBPA degradation.

Moreover, the results of Iasur-Kruh et al. (2010) along the previous findings mentioned above, indicated that TBBPA biodegradation relies on the syntrophic structure of the microbial community with both archeal and bacterial species and that exogenous carbon sources yielding hydrogen, when fermented, enhanced TBBPA degradation. This introduces the first insights into the metabolic mechanism of TBBPA biotransformation under anaerobic conditions and further studies corroborate with the above-mentioned hypothesis. Latic acid may accelerate the debromination process by its utilization as carbon source for microbial growth and the generation of H_2 during its anaerobic bioconversion, acting as electron donor for TBBPA debromination and further BPA degradation (Wei et al., 2018; Lin et al., 2020).

Controversially, Peng et al. (2013) discussed that among different carbohydrates and volatile organic acids, the greatest TBBPA degradation was obtained with formic acid as the main electron donor and carbon source, which is a direct substrate for methanogenesis. However, even though their results suggest that the degradation of TBBPA took place in the methanogenic stage and was favored by formate-consuming microbial groups, at the operational conditions in which the batch runs were tested, it is highly unlikely that more complex substrates (carbohydrates or other volatile fatty acids) were to overcome the prone usage of formate as carbon source for microbial growth. As a consequence, TBBPA biotransformation would occur through reductive debromination rather than H_2-consuming dehalorespiration. In other words, different processes in TBBPA degradation – reductive debromination and dehalorespiratory reactions – will occur if they are favored by the microbial community and operational conditions. Both pathways may yet occur simultaneously in mixed cultures.

Similarly, acetate, which favors methanogenic activity just like formate, was reported to cause a biostimulating effect on TBBPA degradation at the startup of an anaerobic reactor seeded with activated sludge. Lefevre et al. (2019) discussed that the adding acetate would benefit the microbial community only when acetogenesis was not yet occurring. However, with only acetate as organic matter in the system, acetogenesis would not take place in the process whatsoever, as there was no other substrate to be substantially converted to acetate besides the organic content of the seeded sludge itself, which was not reported. The consumption over time of the added acetate was also not

reported, but assumed to be related to TBBPA degradation, which was nearly completed after 34 days. As a prone and easily consumed organic acid, it is unlikely that acetate at an initial concentration of approximately 300 mgAcetate L^{-1} (equivalent COD of 320 mgO_2 L^{-1}) would last throughout this operational period (34 days) in anaerobic conditions. Three years earlier, the same research group (Lefevre et al., 2016) stated that the addition of acetate as a co-metabolic factor delayed the degradation of TBBPA or was not the most suitable substrate for the microbial community.

Many different amendments have been suggested to enhance TBBPA biodegradation (yeast extract, carbohydrates, alcohols, and short chain fatty acids, See Table 1.1). Even though it is well stated that TBBPA degradation is predominantly a co-metabolic process, the feasibility of exogenous organic compounds has not been explicitly related to biochemical mechanisms, with some few exceptions (Iasur-Kruh et al., 2010; Wei et al., 2018; Lin et al, 2020). In addition, the effect of organic amendments depends on many crosslinked factors, such as the operational conditions and the microbial community preferences, which often relies on confusing and controversial discussions.

In summary, the carboxylic formic and acetic acids may favor TBBPA reductive debromination only by providing carbon to microbial growth in a co-metabolic-ATP-dependent process. On the other hand, substrates yielding H_2 production favor TBBPA degradation via the respiratory process, in exergonic reactions, and might occur alongside reductive co-metabolic debromination, by also providing carbon for microbial growth and end products consumed by methanogenic *Archaea*.

Aerobic reactors

Oxic environments often favor micropollutant degradation compared to anaerobic settings, including TBBPA (McAvoy et al., 2016; Ghattas et al., 2017). Activated sludge is the main aerobic system investigated in TBBPA biotransformation as the primary (TBBPA-conversion) (McAvoy et al., 2016; Islam et al., 2018) or the secondary (BPA-conversion) strategy (Ronen and Abeliovich, 2000). The influence of different additives on TBBPA degradation by aerobic consortia has been investigated as much as for anaerobic communities (Table 1.1) and with similar results, suggesting that the degradation of TBBPA in aerobic environments is also driving by co-metabolic processes biostimulated by exogenous carbon sources.

Most of the TBBPA aerobic biotransformation processes focus on isolated and enriched bacteria. The *Ochrobactrum sp.* strain T, which was isolated from an e-waste recycling site, was firstly reported as capable of simultaneously dehalogenating and mineralizing TBBPA by An et al. (2011). This study pioneeringly proved that TBBPA microbial debromination occurs under both aerobic and anaerobic conditions (Ronen and Abeliovich, 2000; Voorderecker et al., 2002). In further studies, it was suggested that ethanol yielded the highest TBBPA degradation efficiency even though the strain's growth was enriched best in the pyruvate-addition system, indicating that ethanol was used primarily as an electron donor rather than carbon source (Zu et al., 2014; Li et al., 2016; Liang et al., 2019 a; Liang et al., 2019 b). In the most recent studies of the group (Liang et al., 2019 a and b), the TBBPA-degrading gene encoding the bromophenol dehalogenase enzyme was identified.

Although many previously mentioned studies on complex and isolated bacterial cultures have concluded that TBBPA is degraded by enzymatic reactions, experiments with isolated *Pseudomonas* sp. and *Pseudoalteromonas* sp. have suggested otherwise. A better understanding of a possible aerobic metabolism involved in TBBPA degradation has been provided by Gu et al. (2016; 2018; 2019) with isolated terrigenous and marine strains and enriched cultures. It is hypothesized that TBBPA conversion is an extracellular process mediated by unknown low-molecular-mass component (LMMC). The authors observed that neither the periplasmic nor the intracellular fraction of the strain is responsible for TBBPA degradation. Instead, 53.22 ± 0.12% of TBBPA (2 mg L^{-1}) was removed by the extracellular fraction after 5 h of cultivation. Whereas TBBPA as the sole carbon source was not degraded by strain fz (*Pseudomonas* sp.), it was concluded that the generation of extracellular active species is not TBBPA-induced and it is a co-metabolic process.

The molecular weight of the active species responsible for TBBPA degradation indicated that it might consist of both aromatic and aliphatic amino acids, containing glycine, proline, and alanine, which might initially complex with Fe (III) to form Fe(III)-oligopeptides. The reduction of Fe(III)−oligopeptides to Fe(II)−oligopeptides might lead to a continuous cycle of Fe(II)/Fe(III)−oligopeptide complexes in the presence of H_2O_2 secreted by strain fz to produce •OH during the process of TBBPA degradation (Gu et al., 2016), giving rise to a microbially driven Fenton reaction.

Further studies with a marine strain (*Pseudoalteromonas* sp.) led to similar results and suggested the same degradation pathways (Beta-scission and debromination), in which approximately 62% of TBBPA-degrading efficiency was found in the extracellular fraction while both the periplasmic and intracellular fractions had a negligible effect on TBBPA removal (Gu et al., 2018). Compared to untreated extracellular fractions, heat and proteinase K treatments showed a suppression of 93 and 44% TBBPA-degrading activity, respectively, implying that both extracellular enzymatic and nonenzymatic active species seemed to play central roles in TBBPA biodegradation (Gu et al., 2018; 2019). The degradation and biotransformation of TBBPA under oxic conditions are also performed by fungi species and their oxidative enzymes (Uhnáková et al., 2011; Brugnari et al., 2018), such as laccase, cellobiose dehydrogenase, and ligninolytic enzymes from spent mushroom compost (SMC) (Yang et al., 2018), but fungi metabolic activity will not be discussed in this review.

Main TBBPA degradation pathway and metabolites in bioreactors

In TBBPA and in many other persistent OMP biotransformation studies, besides the confusion between removal and degradation expressions, the term ‘degradation’ rather than ‘biotransformation’ is often used when TBBPA only was transformed into another hazardous micropollutant (TBBPA sulfate, TBBPA glucoside, TBBPA-MDBPE, TBBPA-MAE, and so on) with no evidence of the extent of degradation. In these cases, considering that the degree of degradation varies from compound to compound, degradation should be evaluated with caution, since TBBPA may only have been transformed into another substance which may be even more toxic and bioaccumulative.

TBBPA biotransformation/degradation has been comprehended as essentially a co-metabolic mechanism, in which microorganisms are able to transform it into products that do not yield energy for microbial growth (Horvath, 1972; Haggblom, 1992), despite studies in which TBBPA was successfully used as the sole carbon source (An et al., 2011; Peng et al., 2013 b; McAvoy et al., 2016; Jiang et al., 2020). Table 1.1 presents the main transformation pathways, end-products, intermediates, and studied conditions regarding TBBPA biotransformation/degradation under anaerobic and aerobic environments, with isolated and mixed bacterial cultures.

Table 1.1 – Main metabolites and operational information in TBBA biodegradation and biotransformation processes.

Concentration	Condition	Medium /Culture	Exogenous carbon source	Pathways	Main transformation products or Intermediates	Reference
25 - 500 µgL^{-1}	Anaerobic	Mixed microorganism biofilm	Glucose	Oxidation, debromination, and dehydrogenation.	2,6-dibromo-4-(1-methyl-1-phenylethyl) phenol (**M6**), 2,6-dibromo-4-(prop-1-en-2-yl) phenol (**M7**),	Fan et al. (2017)
			Glucose and HA		1,3-dibromo-2-methoxy-5-(prop-1-en-2-yl) benzene (**M8**)	Chen et al. (2019)
					1,3-dibromo-2-methoxy-5-(2-(p-tolyloxy) propan-2-yl) benzene (**M9**) 2,6-dibromo-4-(prop-1-en-2-yl) phenyl acetate (**M10**)	Fan et al. (2020)
0.5 mg L^{-1}		Isolated strains	Formate	Oxidation, debromination, and Beta-scission.	2,6-dibromo-4-(1-methyl-1-phenylethyl) phenol (**M6**), 2,6-dibromophenol (**M33**) and phenol (**M11**)	Peng et al. (2014)
		Acclimated sewage sludge	Glucose	Oxidation, debromination, TBBPA-cleavage	less brominated compounds, TBP (**M12**), and oxaloacetic acid (**M13**)	Peng et al. (2017)
10 mg L^{-1}		Sediments from contaminated site	Lactate	Oxidative hydrolysis, debromination, and Beta-scission	BPA (**M5**) further degraded to 4-(allene) phenol (**M14**) and 2,2-bis(4-hydroxyphenyl) propanoic acid (**M15**)	Lin et al. (2020)*
2.5 mg L^{-1}		Activated sludge	Acetate	Reductive debromination	BPA (**M5**)	Lefevre et al. (2016)*
4 mg L^{-1}						Lefevre et al. (2019)
43.5 mg L^{-1}		Stream sediments enriched culture	Ethanol			Arbeli et al. (2006) ; Iasur-Kruh et al. (2010)
50 mg L^{-1}		Anaerobic contaminated sediments	Glucose			Arbeli and Ronen, (2003)*
100 mg L^{-1}		Slurry of anaerobic sediment	Peptone-glucose-yeast extract			Ronen and Abeliovich (2000)*
122.4 mg L^{-1}		Estuarine sediments				Voodereck er et al. (2002)
50 µg kg^{-1} (dw)		Anaerobic sludge, soils, and freshwater sediment	None			MacAvoy et al. (2016)

10.78 mg kg^{-1} (dw)		Mangrove wetlands microorganisms				Jiang et al. (2020)*
20 mg L^{-1}		Paddy soil microorganisms	Lactate			Wei et al. (2018)
0.5 mg L^{-1}		Isolated strains	None	Reductive debromination and benzoyl-CoA reaction	BPA (**M5**), 3, 4-dihydroxymandelic acid (**M16**), beta resorcylic acid (**M17**), acetylbenzoyl (**M18**), acetophenone (**M19**), and oxaloacetic acid (**M13**)	Peng et al. (2013) b
1.1 mg L^{-1}		240-day domesticated biomass	Formate and Yeast extract	-	-	Peng et al. (2013)
2 mg L^{-1}		Isolated strain *Pseudomonas* sp.	yeast extract and HA	-	More polar non-identified components	Ma et al. (2017)
435 µg L^{-1}		Microalgae	None	Debromination, sulfation, glucosylation, and O-methylation	TBBPA sulfate (**M20**), TBBPA glucoside (**M21**), sulfated TBBPA glucoside (**M22**), TBBPA monomethyl ether (**M23**), and TriBBPA (**M2**).	Peng et al. (2014) b
5 mg L^{-1}			Ethanol	Reductive debromination	-	Zu et al. (2014)
3 mg L^{-1}		Isolated strain *Ochrobactrum* sp.T	None	Oxidation, debromination, and O-methylation	TriBBPA (**M2**), BPA (**M5**), 2,4,6-tribromophenol (2,4,6-TBP) (**M12**), 2,6-dibromo-4-(2-hydroxypropan-2-yl) phenol (**M25**), 2,6-dibromo-4-(prop-1-en-2-yl)phenol (**M7**), 2,6-dibromo-4-(2-methoxypropan-2-yl)phenol (**M27**), 2,6-dibromo-4-(propan-2-ylidene)cyclohexa-2,5-dienone (**M24**), and 1-(4-hydroxyphenyl) ethanone (**M26**)	An et al. (2011)
6 mg L^{-1}	Aerobic		Ethanol			Liang et al. (2019) a
10 mg L^{-1}		Isolated strain *Pseudomonas* sp.	Glucose and beef extract	Debromination, Beta-scission, and O-methylation	BPA (**M2**), 4-isopropylene-2,6-dibromophenol (**M7**), 4-(2-methoxylisopropyl)-2,6-dibromophenol (**M27**), 2-bromophenol (**M32**), and 4-(2-hydroxyisopropyl)-2,6-dibromophenol (**M25**)	Gu et al. (2016)*
10 mg L^{-1}		Isolated strain *Pseudoalteromonas* sp.	Peptone and beef extract	Debromination and Beta-scission	TBP (**M12**), 4-isopropylene-2,6-dibromophenol (**M7**), 4-(2-hydroxyisopropyl)-2,6-dibromophenol (**M25**), 4-HBA (**M29**), and 2-BBA (**M30**)	Gu et al. (2018)
10 mg L^{-1}		Isolated strain *Alteromonas macleodii*		Beta-scission, debromination, and nitration routes.	4-(2-hydroxyisopropyl)-2,6-dibromophenol (**M25**), TriBBPA (**M2**), and nitrotribromobisphenol A (**M31**)	Gu et al. (2019)
50 µg g^{-1} (dw)		River sediment	Rhamnolipid	-	-	Chang et al. (2012)

*TriBBPA (**M2**), DiBBPA (**M3**), and MonoBBPA (**M4**) were also detected; All of the studies referenced in this table were conducted under mesophilic temperatures and in the slightly acidic to slightly alkaline pH range. (6.1 to 7.8).

Among anaerobic and aerobic bioprocesses, the main TBBPA biotransformation and degradation routes are based on dehalogenation (reductive and oxidative), Beta-scission, ring cleavage, and O-methylation reactions. Degradation products from BPA breakdown are mainly reported in studies with isolated bacterial strains or acclimated biomass. The studied concentrations are mostly in the mg L^{-1} range, which is much higher than TBBPA environmental occurrence. A variety of exogenous carbon sources has been investigated and many different classes of organic compounds have been suggested as suitable to enhance TBBPA degradation; these results do not suggest a route-pattern on microbial preference as the same pathways have been observed to occur under different conditions and supplemented with distinct carbon sources. Based on these findings (Table 1.1), a diagram of the main biochemical pathways on TBBPA biotransformation/degradation and its respective transformation products was constructed and it is in Figure 1.1. The transformation processes of TBBPA by physical and active-chemicals induced processes have been described by Liu et al. (2018) as well as a succinct microbial degradation pathway scheme.

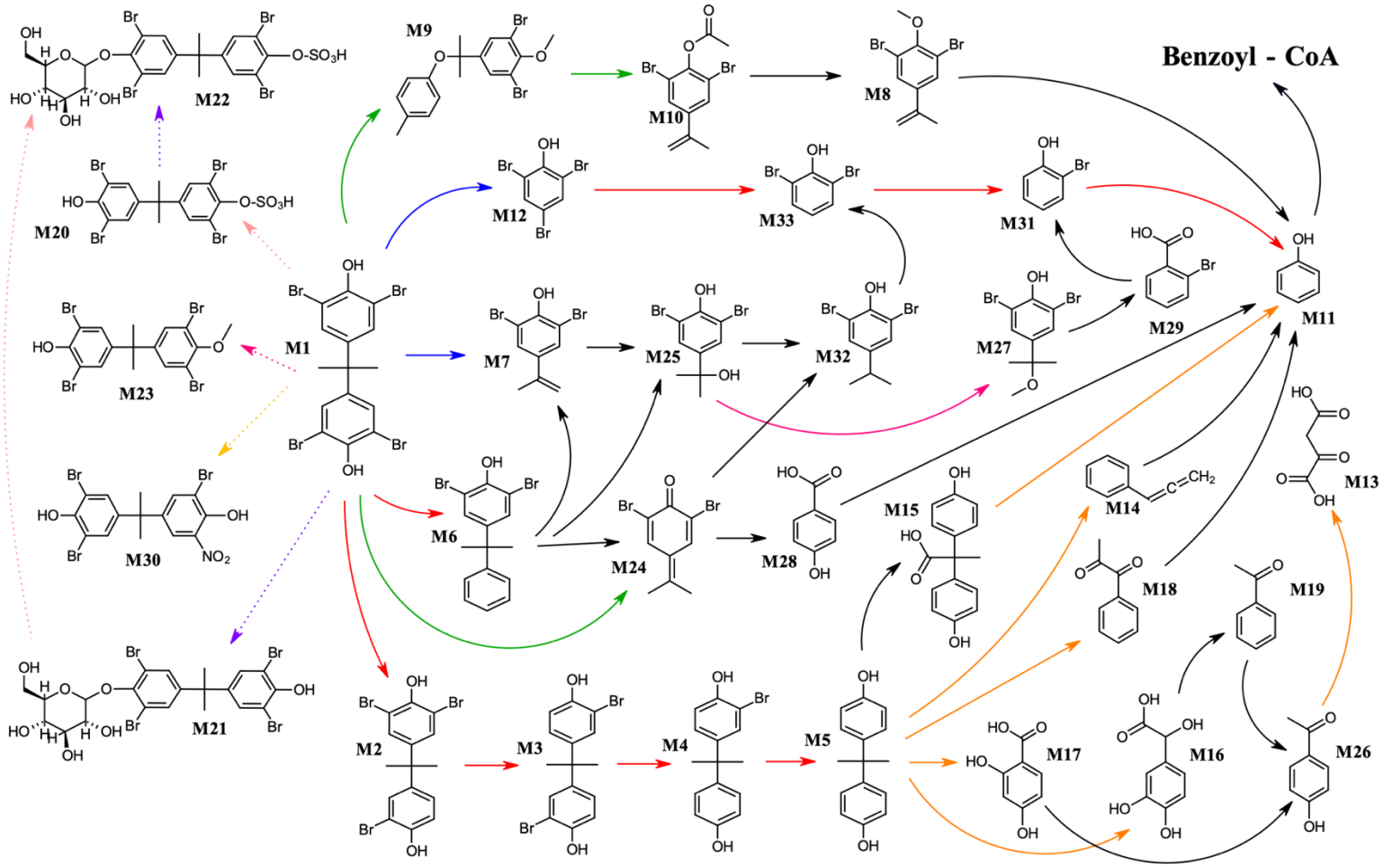

Figure 1.1 – Metabolic diagram of possible degradations and biotransformation routes in biosystems. Continuous and dashed arrows indicate degradation and biotransformation reactions, respectively. Biochemical reactions: Oxidative decomposition (green), Beta-scission (blue), Debromination (red), O-methylation (pink); Nitration (yellow); Sulfation (Light pink/Salmon); Glycolisation (purple); Carbon-carbon bond or ring cleavage (orange). The arrows in black represent multiple or not mentioned reactions such as decomposition, dehydrogenation, hydrogenation, deprotonation, and substitutions.

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Knowledge on microbial breakdown of halogenated aromatic compounds is mostly related to chlorinated rather than brominated chemicals. The degradation pathways to be discussed are somewhat speculative for TBBPA biodegradation, pointing out gaps in our understanding due to the lack of specific in-depth studies on this micropollutant bioconversion. For this reason, the knowledge on other halogenated aromatic compounds should be extrapolated with caution towards TBBPA biodegradation, but it may still elucidate possible biochemical mechanism of TBBPA degradation when related to its main transformation products and environmental conditions. The main degradation and transformation reactions (dehalogenation, reductive debromination, hydroxylation, aromatic ring cleavage, Beta-scission, and O-methylation) will be further discussed.

Dehalogenation

A critical step in TBBPA degradation is the cleavage of the bromide-carbon bond. Similar to other halogenated aromatic compounds, the halogen-carbon cleavage occurs through two main strategies: the bromide is removed as an initial step (via reductive, hydrolytic or oxygenolytic mechanisms) or the bromide removal from the chemical structure occurs after cleavage of the aromatic ring from a phenolic intermediate. In the carbon-halogen bond breakdown achieved by enzyme-catalyzed dehalogenation, the biocatalysis is mediated by specific enzymes, under oxygenolytic, hydrolytic or reductive activity (Hardman, 1991; Haggbolm, 1992; Fetzner, 1998).

The oxidative or oxigenolytic dehalogenation is mediated by mono or dioxygenases in co-metabolic or metabolic reactions. Hydrolytic dehalogenation reactions occur by the substitutive dehalogenation catalyzed via halidohydrolases, in which the hydroxyl group is derived from water rather than molecular oxygen. Lastly, the reductive dehalogenation, which is the most commonly reported step on TBBPA degradation (Table 1.1), occurs in co-metabolic and metabolic (respiratory) processes (Hardman, 1991; Haggbolm, 1992; Fetzner, 1998). The reductive dehalogenation predominance in anaerobic environments is well established for many other halogenated compounds, such as chlorophenols under methanogenic and sulfate-reducing conditions (Hardman, 1991; Haggblom, 1992; Fetzner, 1998). *Aciteobacter, Alcaligenes,* and *Arthorobacter* are some examples of bacterial genera capable of metabolizing haloaromatic compounds by oxygenolytic, reductive, and hydrolytic dehalogenation in enzyme-driven processes, respectively. Furthermore, bacteria may be able to perform all three biocatalytic pathways mentioned above (Hardman, 1991), such as the *Pseduomonas* genus. In fact, the

Pseudomonus sp. strain CBS3 has these three different dehalogenating enzymaticsystems (Hardman, 1991).

Reductive debromination

TBBPA reductive debromination occurring by co-metabolic reactions can be justified by the fact that it is an ATP dependent process and that if only dehalogenation of the substrate is taking place and the aromatic ring remains intact, no carbon is provided for microbial growth (Haggblom, 1992; Fetzner, 1998). However, besides the reductive debromination accomplished by co-metabolism, reductive dehalogenation as a respiratory process (dehalorespiration) may take place on halogenated aromatic compound degradation, in a process where TBBPA would be used as electron acceptor in an exergonic dehalogenation reaction, yielding energy that could be used for bacterial benefit (El Fantroussi et al. 1998; Fetzner, 1998; Zhang et al., 2005). Reductive debromination may also involve specificity towards the ring position of the halogen (*ortho*, *meta*, and *para*) and to the halogen atom to be removed (Br, Cl, or I), but not necessarily to the type of the halogenated compound. Due to these specificities, reductive dehalogenation activities are most likely catalyzed by distinct organisms with different dehalogenating enzymes (Mohn and Tiedje, 1992).

In fact, metabolic reductive dehalogenation rates in general are several orders of magnitude higher than co-metabolic rates (Fetzner, 1998). Anaerobic bacterial species may utilize several electron donors and acceptors in a respiratory system that involve hydrogenases, reductive dehalogenases, and electron carriers mediating electron transfer between them (Fetzner, 1998), with relaxed substrate specificity, meaning that a bacterial group may degrade a variety of halogenated compounds using different electron donors. In an analogous way, in chlorinated compound degradation, the sulfate-reducing bacterium *Desulfomonile tiedjei* have the capacity of using formic acid or H_2 as an electron donor and 3-chlorobenzoate as a terminal electron acceptor in a respiratory process (Fetzner, 1998) and the chemiosmotic coupling of reductive dechlorination and ATP synthesis has been demonstrated (Mohn and Tiedje 1991; Fetzner, 1998).

In light of these facts, fermentation of organic compounds such as fatty acids and carbohydrates would provide the electrons needed for TBBPA reductive dehalorespiration from H_2 as an electron donor or even other intermediates (Fetzner, 1998; Zhang et al., 2005). These facts may explain why

so many different exogenous carbon sources (Table 1.1) have been indicated as the most suitable to support TBBPA degradation under anaerobic conditions.

Exogenous organic substances may positively influence TBBPA degradation by providing both carbon and energy source for microbial growth, enabling co-metabolic (with no benefit for bacterial growth) and metabolic (by providing electrons/energy such as H_2) reductive debromination. Most importantly, reductive dehalogenation linked to carbon metabolism may occur in TBBPA degradation by the reductive removal of the bromides followed by assimilation of the corresponding carboxylic acids (Figure 1.1). It is hypothesized that in complex microbial communities, both processes (co-metabolic and respiratory) may occur simultaneously in TBBPA degradation even at environmentally relevant concentrations, but this remains unexplored.

Hydroxylation, Aromatic ring cleavage, Beta-scission, and O-methylation

The prevailing pathways for both aerobic and anaerobic degradation of TBBPA is that the halogen substituents are removed one by one through either hydroxylation or reductive debromination, and then the aromatic ring is cleaved (Heider and Fuchs, 1997). However, the TBBPA degradation pathways proposed by many authors (Gu et al., 2016; 2018; 2019; An et al., 2011; Liang et al., 2019; Fan et al., 2017; 2020) are in contrast, in which the bromide atoms are removed only after ring cleavage.

Aromatic ring cleavage is a critical step in the degradation of halogenated aromatic compounds and intermediates, such as catechols, which are readily attacked oxidatively in aerobic degradation. Under anaerobic environments, in the cleavage of aromatic rings, the carbon-carbon bonds are broken by reduction and the ring is subsequently opened hydrolytically. The most common central intermediate in anaerobic aromatic metabolism is benzoyl-CoA, for which the common key enzyme for ring reduction in these metabolic routes is benzoyl-CoA reductase (dearomatising) (Heider and Fuchs, 1997; Hardwood et al., 1998).

The hydroxylation process, an oxidation reaction in which carbon–hydrogen (C-H) bond oxidizes into carbon–hydroxyl bond (C-OH), may be one of the mechanisms undergoing TBBPA biotransformation (M15, M16, M17, M25 – Table 1.1). This process is mediated by enzymes (i.e. phenol hydroxylases) that may be produced by bacteria that had no history of exposure to halogenated phenols and, therefore, have a broad substrate specificity (Haggblom, 1992). Monoxygenases and dioxygenases are essential enzymes for the hydroxylation and cleavage of

aromatic ring structures (Haggblom, 1992; Heider and Fuchs, 1997). For chlorocatechols, two isofunctional enzymes were related to the process, pyrocatechase type I (catechol dioxygenase), which is highly specific for catechol, and pyrocatechase type II, which does not require substrate specificity and had high activity for chlorocatechols. Furthermore, enzymes acting on ring cleavage may express specificity to *ortho*-, *meta*-, or *para*-cleavage activity (Horvath, 1970; Haggblom, 1992; Zhang et al., 2005).

Beta-scission reaction is often related to TBBPA biodegradation under both aerobic and anaerobic conditions (Table 1.1). The microorganisms and/or enzymes driving this process remains unknown and its occurrence on TBBPA break down is solely based on the transformation products identified. Beta-scission acts on the cleavage of a carbon–carbon bond and may generate from BPA, TBBPA or a less brominated bisphenol a variety of transformation products such as 2,6-dibromophenol and 4-(1-methylethenyl) phenol (Peng et al., 2014; Barontini et al., 2004).

O-methylation on halogenated compounds is the methylation of hydroxyl groups, which has been reported under aerobic conditions for TBBPA degradation (Peng et al., 2014; Gu et al., 2016; 2018; Liang et al., 2019). The ability to O-methylate chlorophenols is widespread in nature and is performed by different bacterial genera and fungi species. Even though a wide range of halogenated phenolic compounds are O-methylated by bacteria, indicating that the enzymes are not very specific, differences in the response to chlorophenol isomers have been observed (Haggblom 1992).

The halo-compounds produced by O-methylation are often less toxic to bacteria and fungi than the corresponding chlorophenols or bromophenols (Ruckdeschel et al., 1987; Peng et al. 2014). Associated to the fact that O-methylation enzymes are not specific, this reaction might function as a detoxification mechanism. O-methylation catalyzed by O-methyltransferases successively transfers a methyl group to the two hydroxyl groups of TBBPA (Cho et al., 2008; Xu et al., 2018). The bottom side of the process is that the replacement of the OH groups of TBBPA by MeO groups further increases the lipophilicity of TBBPA, turning it into a more bioaccumulative micropollutant.

Enzymatic and Meta-omic studies

Micropollutants degradation by enzymatic processes have been discussed and investigated recently. Besides enzymes from a few isolated strains (Yi et al., 2017; Chen et al., 2019), there is

no comprehensive knowledge of TBBPA bioconversion by native enzymes from complex microbial communities. Additionally, metabolic conversion of xenobiotics in general by microbial cells involves a variety of interactions and changes in functional proteins rather than a single effective enzyme.

Very few studies have tried to extract native enzymes from complex inoculum in order to elucidate the biotransformation mechanism undergoing micropollutants bioconversion in natural environments. None have been tested for TBBPA or other brominated flame retardants to date. Krah et al. (2016) and Gonzalez-Gil et al. (2019) have extracted native enzymes from activated and anaerobic sludge, respectively, and evaluated the cell-free lysates on the biodegradation of a variety of OMPs. However, the full variety of enzymatic activity of the complex sludges and the tested conditions could not be restored. The authors stated that the vast number of enzymes that could participate in the process, their interactions, and the complexity of the micropollutants make it difficult to elucidate the enzymatic pathways involved in OMP biotransformation.

Aside from the role of enzymes on TBBPA biotransformation, its degradation has been associated with an extracellular nonenzymatic process (Gu et al., 2016; 2018), driven by low-molecular-mass by a biogenic Fenton-like reaction mediated via extracellular H_2O_2 and Fe(III)-oligopeptide complexes rather than by enzymes.

Meta-omics studies characterize the entire pool of genes in a complex microbial community, thus establishing statistical associations with biotransformation parameters (Krah et al., 2016). Metagenomics, metaproteomics, and metabolomics studies evaluate the functional diversity at the genetic, translation, and metabolite level, respectively. These molecular technologies give in-depth understanding of structure and functional aspects of microbiome and uncovers deeper insights into microbial activities with respect to their genes, proteins, mRNA expression, enzymes, and metabolic pathways in regard to OMPs degradation. Meta-omic studies would allow the identification of genes encoding degradative enzymes for the bioconversion of flame retardants, for example. Nonetheless, none have been performed regarding the bacterial conversion of TBBPA or other brominated flame retardants to date, with a few exceptions (Yu et al., 2020). A comprehensive review on meta-omic technologies for the characterization of xenobiotics-degrading microorganisms has been recently published (Mishra et al., 2021).

Meta-omics analysis suggests that *Phanerochaete chrysosporium* could biotransform TBBPA under anaerobic conditions by oxidative cleavage, reductive debromination, oxidative

hydroxylation and glycosylation pathways by the expression of oxidoreductase, cytochrome P450 monooxygenase, glutathione S-transferases, and UDP-glycosyltransferases, which were up-regulated in the presence of the micropollutant (Chen et al., 2019 b).

For the bioconversion of other flame retardants, such as diphenyl ether (DE) and its 4-monohalogenated derivatives (4-HDE) (Pacholak et al., 2018), 2,4,6-tribromophenol (TBP) (Liang et al., 2019), and triphenyl phosphate (TPhP) (Wang et al., 2019) by specific strains (*Pseudomonas plecoglossicida* IsA (IsA) and *Pseudomonas fluorescens* B01 (B01), *Bacillus* sp. GZT, and *Sphingomonadaceae* family, respectively), metagenomic and proteomic analysis led to specific metabolic pathways, transformation products, and the comprehension of co-metabolic factors. However, isolated or enriched laboratory cultures usually do not provide reliable information about the activity of the enzymes and genes actually involved in micropollutant degradation and may not be transferable from laboratory to environmental settings.

TBBPA effect on microbiota: the genera related to its degradation and their enzymatic activity

The ability to biotransform OMPs is generally attributed to specific strains, however TBBPA reductive debromination and further degradation seems to require a consortium of species with different metabolic preferences and substrate. It has been suggested that syntrophic relations among TBBPA degrading and non-degrading microorganisms would explain greater efficiency on TBBPA degradation by co-cultures rather than a few isolated strains (Iasur-Kruh et al., 2010; Peng et al., 2014; Lefevre et al., 2016; Lefevre et al., 2019). Most of the enzymatic activities on OMPs degradation are non-specific substrate processes (Mohn and Tiedje, 1992; Haggblom, 1992; Fetzner, 1998), in which the enzymes might act on different substances based on operational conditions and substrate availability. In experiments on TBBPA degradation, very few enzymes or non-enzymatic extracellular substances have been identified. However, these reaction-mediators are most likely similar to the ones identified for other halo-aromatic or halo-phenolic substances.

The production of these enzymes may be intentionally stimulated by controlled and optimized conditions (Bains et al., 2019) and by the exposure to the contaminant(s). For example, dissolved oxygen perturbations may cause oxidative stress in a mixed microbial culture generated by high concentrations of intracellular reactive oxygen species (ROS) (O_2, H_2O_2, and OH•) and, as a consequence, induce gene expression encoding the synthesis of oxidoreductases (peroxidases and

cytochromes). The results of this is a greater efficiency on the degradation of OMPs by these enzymes (Bains et al., 2019). Similarly, the stimulus for gene encoding enzymatic expression towards TBBPA (or other micropollutant) may be achieved by the exposure to TBBPA (Iasur-Kruh et al., 2010; Liang et al., 2019 b, c).

Laccases, cytochrome P450 monooxygenase, glutathione S-transferases, UDP-glucosyltransferase, O-methyltransferase and other oxidoreductases are examples of non-substrate-specific fungi and bacterial enzymes efficient in degrading TBBPA and BPA (Brugnari et al., 2018; Chen et al., 2019 b). Some of those enzymes are produced by both anaerobic and aerobic bacterial genera and are responsible for oxidative hydroxylation, reductive debromination, glycosylation, O-methylation, and oxidative cleavage reactions (Haggblom, 1992; Fetzner, 1998).

Acetate kinase (AK) is a key enzyme in methane production and is also able to transform BPA alongside many other OMPs under methanogenic conditions (Gonzalez-Gil et al., 2017). Its action towards OMP bioconversion is determined by the chemical structure, and for AK specifically, only compounds that contain a carboxyl or hydroxyl group and have moderate steric hindrance were enzymatically transformed (Gonzalez-Gil et al., 2017), demonstrating that even though BPA may accumulate as an end-product in TBBPA-degrading experiments, it does not mean that this subproduct may not suffer extended degradation in anaerobic environments. As no transformation product from BPA was evaluated, it is difficult to conclude by which means AK acts on this micropollutant biotransformation.

Dehalogenation is a critical step on TBBPA conversion, as previously mentioned. For most chlorinated aromatic compounds, bacteria have evolved several strategies for the enzyme-catalyzed dehalogenation and further degradation in aerobic and anaerobic medium, which may be similar to possible degradation processes on brominated aromatic compounds, such as TBBPA (Mohn, 1991; Haggblom, 1992; Fetzner, 1998). Some of these enzyme-catalyzed dehalogenation reactions are oxidative dehalogenation (with mono or dioxygenase), dehydrohalogenase-catayzed dehalogenation, substitutive dehalogenation in hydrolytic process (by halidohydrolases), dehalogenation by halohydrin hydrogen-halide lyases, and reductive dehalogenations as co-metabolic or respiratory processes (Fetzner, 1998).

There are no studies on the genomic and enzymatic activity of TBBPA-degradading mixed communities and only a few studies on isolated strains (Gu et al., 2016; 2018; Xie et al., 2018; Xu et al., 2018; Liang et al., 2019 a; 2019 b). The genomic study of the isolated strain (*Ochrobactrum*

sp), capable of debrominating TBBPA by reduction and mineralizing by oxidation, showed genes that encode an enzyme complex containing dehalogenases, monooxygenases, oxidoreductases, decarboxylases, hydroxylase, oxidase and hydrolase, which are functionally related to the degradation of environmental contaminants, including bromo and chlorinated phenolic and non-phenolic compounds. In further studies, the dehalogenase responsible for 78% of TBBPA degradation was purified and designated as bromophenol dehalogenase and gene expression was up-regulated in the presence of TBBPA (An et al., 2011; Li et al., 2016; Liang et al., 2019 a, 2019 b).

Gene expression and enzymatic activity towards TBBPA degradation may be stimulated by the exposure to TBBPA. Under anaerobic conditions, the genes related to the hydrogenase enzyme and dichlorination functions increased 100-fold under TBBPA exposure conditions in the *Anaeromyxobacter* genus (Xie et al., 2018), indicating TBBPA stimulation effects and that dichlorination functions are not specifically related to a narrow class of chemical (chloro-aromatic) but rather a broader classification (halo-aromatic compounds).

Rather than enzymatic activity, the genera *Pseudomonas* (Gu et al., 2016) and *Pseudoalteromonas* (Gu et al., 2018) genomic information associated TBBPA conversion with biogenic Fenton-like reactions mediated by extracellular Fe(II)-oligopeptide complexes and the upregulation of genes encoding Na^+-translocating NADH-quinone oxidoreductase and L-lysine-ε-oxidase, respectively, supported by extracellular O_2 and H_2O_2 production.

Comprehending the metabolism of isolated strains on TBBPA degradation may provide information on enzymatic activity, cofactors, and chemical mechanisms. However, it does not represent the biocatalytic potential of complex microbial cultures nor how ecological factors (temperature, pH, substrate availability, nutrients and interferents) will affect it under natural environments. Meta-omics studies on microbial consortia rather than isolated strains would hopefully elucidate what really drives TBBPA and other OMP degradation of hazardous materials and by which means. In addition, mixed cultures have advantages in the transformation/degradation of complex compounds because of their richness in species and enzymes, often collaborative, enabling the performance of more difficult tasks and endurance under severe contamination conditions compared to monoculture. The ability to biotransform TBBPA is not an exclusive feature of few strains, observed by the identified enzymes, the metabolic routes, and the variety of

identified genera (Table 1.2), which are mostly related to the degradation of several compounds, haloaromatic or not.

In addition to the metabolic performance of bacteria, TBBPA may cause modifications in the taxonomic distribution of mixed cultures, which is a controversial topic probably due to the wide range of studied concentrations, operational conditions, and seed-inoculum. For Lefevre et al. (2016), repeated exposure to high concentrations of TBBPA did not cause significant temporal shifts in the microbial community, while other authors stated that besides the TBBPA significant role in the changes of microbial community structure and composition (Iasur-Kruh et al., 2010; Xie et al., 2018; Chen et al., 2019), it is an intracellular metabolism disruptor (Wang et al., 2019). Moreover, it was hypothesized that mainly Gram-positive bacteria rather than Gram-negative bacteria are inhibited by TBBPA exposure (Xie et al., 2018) and that the bacterial ability of dehalogenation is TBBPA-dependent (Iasur-Kruh et al., 2010). In comparison to Gram-positive bacteria, the Gram-negative bacterial phyla have an outer membrane which confers increased resistance to both hydrophobic antibiotics and dyes (Harrop, 1989).

In the past 20 years, many bacterial genera have been associated to TBBPA degradation activity. The main bacterial genera related to TBBPA biotransformation and degradation is summarized in Table 1.2, alongside its taxonomic classification. Bacterial identification indicates that even though microorganisms related to TBBPA conversion belongs to many different phyla, there is a predominance of the *Proteobacteria* phylum. In fact, 24 out of 33 identified genera belong to Gram-negative phyla (*Bacterioidetes, Spirochaetes*, and *Proteobacteria*), which corroborate Xie et al. (2018) statement. In long-term incubations of contaminated river sediments, *Proteobacteria* was the predominant phylum (46% to 60%) and hardly changed with incubation time (Wang et al., 2021). The gram-positive bacteria belong to the phyla *Firmicutes* (*Bacilli* and *Clostridia*) and *Actinobacteria* (*Rhodococcus*).

Among these gram-positive bacteria, *Rhodococcus* has been associated to the degradation of many chlorinated aromatic and aliphatic compounds in anaerobic and aerobic environments through haloalkane halidohydrolases, monooxygenases, and other enzyme-catalyzed hydrolytic and o-methylating activity (Hardman, 1991; Mohn and Tiedje, 1992; Fetzner, 1998). Many of the gram-negative bacteria mentioned in Table 1.2 have been associated to OMP degradation, but *Pseudomonas* is the mostly reported genus associated with a variety of OMPs via enzymatic-mediated reactions (reductases, hydrogenases, dehalogenases, dioxygenase, and P-450-type

enzymes) (Hardman, 1991; Mohn and Tiedje, 1992; Fetzner, 1998) and non-enzymatic reactions (Gu et al., 2016).

Table 1.2 – Bacterial genera associated with TBBPA biodegradation and biotransformation.

Phylum	Class	Order	Family	Genus	Reference
Bacteroidetes	*Sphingobacteriia*	*Sphingobacteriales*	*Chitinophagaceae*	*Parasegetibacter*	Li et al. (2016)
				Flavisolibacter	Xie et al. (2018)
	Flavobacteria	*Flavobacteriales*	*Flavobacteriaceae*	*Flavobacterium*	Peng et al. (2012); Jiang et al. (2020); Peng et al. (2017); Yang et al. (2016)
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	Yu et al. (2019); Lin et al. (2020); Yang et al. (2018); Chang et al. (2012); Yang et al. (2016); Cheng et al. (2019b); Liang et al. (2019c)
		Lactobacillales	*Streptococcaceae*	*Streptococcus*	Peng et al. (2014); Lefevre et al. (2016); Yang et al. (2016)
			Carnobacteriaceae	*Trichococcus*	Fan et al. (2017); Fan et al. (2020); Chen et al. (2019)
	Clostridia	*Clostridiales*	*Peptococcaceae*	*Cryptanaerobacter*	Lin et al. (2020)
				Dehalobacter	Zhang et al. (2013); Lefevre et al. (2016)
			Eubacteriaceae	*Acetobacterium*	
			Clostridiaceae	*Clostridium*	Peng et al. (2017); Cheng et al. (2019)b
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Brucellaceae*	*Ochrobactrum*	An et al. (2011) ; Zu et al. (2014); Liang et al. (2019a; 2019b), Li et al. (2016)
		Alphaproteobacteria	*Caulobacterales*	*Phenylobacterium*	Li et al. (2016)
		Sphingomonadales	*Sphingomonadaceae*	*Sphingomonas*	Li et al. (2014); Li et al. (2016); Yang et al. (2016); Yang et al. (2018); Cheng et al. (2019)b
				Erythrobacter	Yang et al. (2018)
	Gammaproteobacteria	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	Peng et al. (2012); Peng et al. (2014); Yu et al. (2019); Lefevre et al. (2016); Jiang et al. (2020); Yang et al. (2016); Yang et al. (2018); Peng et al. (2017); Peng et al. (2017); Xie et al. (2018); Gu et al. (2016)
		Enterobacteriales	*Enterobacteriaceae*	*Citrobacter*	Lefevre et al. (2016); Cheng et al. (2019)
		Alteromonadales	*Pseudoalteromonadaceae*	*Pseudoalteromonas*	Gu et al. (2018); Gu et al. (2019)

			Colwelliaceae	*Thalassomonas*	
			Alteromonadaceae	*Alteromonas*	Gu et al. (2019)
			Shewanellaceae	*Glaciecola*	
				Shewanella	Wang et al. (2013)
		Rhodocyclales	*Zoogloeaceae*	*Azoarcus*	Fan et al. (2017); Fan et al. (2020); Jiang et al. (2020)
	Betaproteobacteria	*Burkholderiales*	*Comamonadaceae*	*Comamonas*	Peng et al. (2012); Lefevre et al. (2016); Peng et al. (2013)b; Peng et al. (2017)
			Alcaligenaceae	*Achromobacter*	Peng et al. (2012); Peng et al. (2017)
			Burkholderiaceae	*Limnobacter*	Gu et al. (2019)
			Pelobacteraceae	*Pelobacter*	Iasur-Kruh et al. (2010)
		Desulfuromonadales	*Geobacteraceae*	*Geobacter*	Zhang et al. (2013); Lefevre et al. (2016); Jiang et al. (2020); Yang et al. (2016); Cheng et al. (2019)b
	Deltaproteobacteria	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*	Lefevre et al. (2016); Lin et al. (2020); Yang et al. (2018)
		Myxococcales	*Myxococcaceae*	*Anaeromyxobacter*	Lefevre et al. (2016); Xie et al. (2018); Cheng et al. (2019) b
	Epsilonproteobacteria	*Campylobacterales*	*Campylobacteraceae*	*Sulfurospirillum*	Zhang et al. (2013); Lefevre et al. (2016)
	Acidithiobacillia	*Acidithiobacillales*	*Thermithiobacillaceae*	*Thermithiobacillus*	Li et al. (2016)
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Nocardiaceae*	*Rhodococcus*	Yang et al. (2018); Yang et al. (2016); Chang et al. (2012); Xu et al. (2018)
Spirochaetes	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	*Sphaerochaeta*	Iasur-Kruh et al. (2010); Yang et al. (2018).

Concluding remarks

The relevance of fundamental research on how a micropollutant is biologically converted is the first step to apply biochemical knowledge on industrial processes. Comprehending TBBPA biodegradation may lead to a better understanding in terms of the environmental fate of other flame retardants and optimization of wastewater treatment biotechnologies.

Most of the mechanisms that explain TBBPA dehalogenation and further degradation with ring cleavage is extrapolated from previous studies on other halogenated aromatic compounds or elucidated by few enriched and isolated bacterial species. It seems that the degradation of TBBPA is catalyzed by physiologically diverse organisms in different environmental settings. Dehalogenating bacterial communities, for example, appear to vary significantly in their composition and respond differently to environmental factors. The ability to metabolize several electron acceptors indicates that dehalogenation is not a substrate-specific reaction and that most of the organisms may achieve this conversion via syntrophic relationships and cometabolic reactions. This highlights the urge to further researches on TBBPA biodegradation by microbiome at environmentally relevant concentrations.

Enriched populations often demonstrate substrate specificity and singular interactions with ecological factors. Studies on isolated strains provide crucial knowledge on the chemical mechanisms involved in TBBPA degradation, enzymes and cofactors related to biocatalytic reactions, and how these organisms are benefited from TBBPA conversion. However, the extrapolation of microbial mechanisms to environmental settings is limited as biochemical pathways, enzymatic activity, cofactors, and environmental interference on TBBPA degradation by natural occurring microbial communities remains scarce and underexplored. Tracing back gene expressions from isolated strains in enriched media to its occurrence in microbiomes could confirm that the degradation mechanism is the same (under both conditions) or induced by high concentrations exposure. Furthermore, the operational conditions studied on TBBPA degradation (TBBPA concentration, specific carbon sources, pH values, temperature and other controlled operational factors) led to results that are not always consistent. Controlled and simplified experimental settings provide knowledge on how microorganisms may transform TBBPA and how the process may be optimized. However, controlled operational factors may not represent the composition and physical-chemical state of real contaminated wastewaters. Therefore, the results on TBBPA degradation in those circumstances may not represent the actual fate of this chemical

in natural ecosystems and lead to different outcomes based on each microbiome. Instead, it is suggested that experimental conditions should consider simulating the environmental matrices in which TBBPA is present: industrial wastewaters, domestic sewage, contaminated soils, and e-waste leachate effluents.

In light of this, future meta-omics studies of TBBPA-degrading complex microbial communities might bring new insights into its degradation under environmentally relevant conditions. So far, the studied conditions, the concentrations, and the identified transformation products indicate that TBBPA degradation is driven by diverse and cooperative microbial communities in co-metabolic and metabolic mechanisms that may occur simultaneously. Nonetheless, the understanding of TBBPA conversion at the level of enzyme−chemical interactions is limited. Most of the identified TBBPA-degrading enzymes have a relaxed substrate specificity, catalyzing several halogenated and non-halogenated analogs or related compounds. Thus, the bacterial consortia acting on TBBPA degradation may not be necessarily designed for degrading halo-aromatic compounds but possess an enzymatic machinery surely capable to do so. In this sense, metaproteomic studies with labelled isotopes could give in-depth knowledge on the degradation of micropollutants occurring in bioreactors. To have detailed answers on what drives TBBPA biodegradation, some questions should be addressed in profound studies: 1) how TBBPA is biodegraded in environmental matrices at environmentally relevant concentrations, 2) how syntrophic microorganisms enzymatically catalyze TBBPA conversion, and 3) what the association is between TBBPA biotransformation and specific gene products in complex microbial communities.

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Chapter 2

Application of Dispersive Liquid–Liquid Microextraction Followed by High-Performance Liquid Chromatography/ Tandem Mass Spectrometry Analysis to Determine Tetrabromobisphenol A in Complex Matrices

Abstract: An accurate and sensitive ultrasound dispersive liquid-liquid microextraction (US-DLLME) followed by high-performance liquid chromatography (HPLC) separation coupled with Electrospray Ionization tandem Mass Spectrometry detection (HPLC-ESI-MS/MS) method to determine Tetrabromobisphenol A (TBBPA) in complex environmental matrices is proposed. The miniaturized procedure was used to extract and quantify the analyte in domestic sewage (DS), anaerobic sludge (AS), and aquatic test-organism species, *Daphnia magna* (DM) and *Chironomus sancticaroli* (CS), which are standardized organisms for ecotoxicity bioassays. Limits of detection (LOD) of 2 ng L^{-1} (DS), 2 ng g^{-1}(AS), 0.25 ng g^{-1} (DM), and 5 ng g^{-1} (CS) were obtained. TBBPA was determined in domestic sewage and anaerobic sludge from an anaerobic batch bioreactor at a concentration of 0.2 ± 0.03 μg L^{-1} and 507 ± 79 ng g^{-1}, respectively. In DM and CS exposed to TBBPA in an acute toxicity bioassay, the micropollutant accumulated at 3.74 μg g^{-1} and 8.87 μg g^{-1}, respectively. The proposed method is a simple and cost-effective tool to determine TBBPA environmental occurrence and biomagnification potential compared to conventional extraction methods. To the best of our knowledge, this is the first liquid-liquid miniaturized extraction method applied to *Daphnia magna* and *Chironomus sancticaroli.*

Keywords: Domestic sewage; anaerobic sludge; *Daphnia magna; Chironomus sancticaroli*; Bioaccumulation; DLLME-HPLC-ESI-MS/MS.

Introduction

Tetrabromobisphenol A (TBBPA) is the most common flame retardant applied to electric and electronic equipment, epoxy resins, and plastic products to meet fire safety requirements. This organic compound is an environment micropollutant and has been detected in air, dust, sediment, biota, and water. In water bodies, its concentrations range from undetectable values to 30,000 ng L^{-1} (Yang et al., 2012; Ni and Zeng, 2013; Wang et al., 2014; Zhou et al., 2014; Qu et al., 2016;

Daso et al., 2017; Rothenbacher and Pecquet, 2018). Although the toxic effects of TBBPA on living beings are not yet defined, some studies indicate that this micropollutant may cause endocrine changes in organisms, it may be associated with the development of cancer in the uterus of rats, maternal transmission between fish generations, and genetic mutation in frogs (Veldhoen et al., 2006; Nyholm et al., 2008; Yang et al., 2012; NTP, 2014).

Sample preparation is one of the most important steps to identify and quantify micropollutants in environmental samples including the elimination of matrix interferents, preconcentration, and isolation of analytes. Extraction and clean-up procedures that align the Green Analytical Chemistry (GAC) principles with good sensitivity, speed, precision, accuracy and efficiency, providing low LOD values and relative recoveries have been developed over the past decades (Rutkowaska et al., 2016; Kabir et al., 2017; Primel et al., 2017; Burato et al., 2020). Minimizing or eliminating toxic organic solvent consumption, reducing sample volume, and extraction time while maintaining or enhancing extraction efficiency are some of the concerns of GAC (Burato et al., 2020). Even though Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are the best conventional techniques for sample preparation, the aim of using minimum solvent and sample volumes lead to miniaturized concepts, such as the ultrasound assisted liquid-liquid microextraction (US-DLLME), which are more environmentally friendly (Rutkowaska et al., 2016; Kabir et al., 2017; Burato et al., 2020).

To determine TBBPA in complex matrix samples, extraction and clean-up methods are still based on non-miniaturized techniques. Analytical methods to determine TCBPA, PeBP, BPA, TBBPA, HBCD, PBDE, and bromophenols in complex solid and aqueous samples based on using a larger volume of organic solvent, SPE cartridges, time-consuming steps throughout the procedure, and complex apparatus compared to DLLME procedures have been extensively reported (Deceuninck et al., 2014; Chen et al., 2016; Zhang et al., 2016; Li et al., 2017; Chi et al., 2017). For environmental complex samples, the DLLME technique may be combined with other extraction and/or clean-up procedures depending on the sample's nature. Generally, more attention has been given to aqueous environmental samples regarding the application of DLLME; solid ones are still underexplored.

Domestic sewage and anaerobic sludge are important matrices for determining the environmental occurrence of hazardous organic chemicals due to the fact that wastewater treatment plants (WWTP) receive contaminants through industrial, hospital, and domestic sewage discharges,

and mostly do not effectively remove and/or degrade micropollutants, which contaminate water bodies and ecosystems (Gorga et al., 2013). Predicting the possible impact of substances in the environment and their interaction with organisms is one of the aims of ecotoxicological bioassays. Using invertebrates' organisms in these studies is highly relevant as they are primary consumers in aquatic ecosystems and are used as prey for high-level consumers (Chaumot et al. 2014; Baun et al. 2008; Newman 2008; Cattaneo et al. 2009; O'Brien et al., 2016). *Daphnia magna* Straus 1820 and *Chironomus sancticaroli* Strixino & Strixino 1981 are a freshwater aquatic invertebrate representative species of the water column and benthic habitat, respectively. They are representative of zooplankton organisms and recommended for ecotoxicological tests to assess water quality (Müller, 1980; Koivisto, 1995; Baumann et al., 2014; Besseling et al., 2014; Colombo-Corbi et al., 2017; Horton et al., 2018; Richardi et al., 2018; Corbi et al., 2019; Bernegossi et al., 2019; Dornfeld et al., 2019). The effective concentration (EC) of TBBPA which affects 50% of daphnid mobility was less than 1 mg L^{-1} (48-h EC50 of 0.6 mg L^{-1}) (Waaijers et al., 2013). Even though the concentration of TBBPA in the body fluid of D. magna has been reported (Choi et al., 2020), to the best of our knowledge, there is no report on analytical methods based on DLLME using the whole organism for determining TBBPA bioaccumulation. In the Chironomidae family, TBBPA lethal concentration (LC) which affects 50% of the organisms in water was determined as LC50 of 0.13 mg L^{-1} for the *Chironomus tentans* species (14-day exposure). In sediments, the lowest observed effect concentration (LOEC) for larvae emergence ratio and development time was determined as 250 mg TBBPA kg^{-1} of dried sediment (28-day exposure) (EPA, 2005). Moreover, there is no published information regarding the detection or bioaccumulation of TBBPA in the *Chironomus sancticaroli* species. Several studies have assessed the potential of bioaccumulation of flame retardants in biological matrices (animals and plants) in the laboratory and field conditions (Bragigand et al., 2006; Sun et al; 2007; Gustafsson et al., 1999; Tian and Zhu, 2011; Wu et al., 2011; Mansouri et al., 2012) and it is the first step to determine bioconcentration and biomagnification capacity of hazardous substances (Law et al., 2006; Sormo et al., 2006; Choo, Lee and Oh, 2019).

Thus, this study applies a US-DLLME technique followed by high-performance liquid chromatography (HPLC) separation coupled to electrospray ionization tandem mass spectrometry (ESI-MS/MS) detection to identify and quantify TBBPA in domestic sewage (DS), anaerobic sludge (AS), and in the aquatic invertebrates *Daphnia magna* (Dm) and *Chironomus sancticaroli*

(Cs) species. Therefore, the established method was used to determine TBBPA in real samples. To the best of our knowledge, this technique has not been applied to determine TBBPA in the studied matrices and there is no report on TBBPA bioaccumulation in *Chironomus sancticaroli.*

Material And Methods

Chemicals and materials

Tetrabromobisphenol A (TBBPA) (TBBPA, 4,40-Isopropylidenebis (2,6-dibromophenol), 97% purity, CAS 79-94-7) and the stable isotope labeled internal standard ($^{13}C_{12}$-TBBPA) were purchased from Sigma-Aldrich (Brazil) and from Wellington Laboratories (Guelph, Canada), respectively. All organic solvent methanol (JT Baker), acetonitrile (JT Baker), tetrahydrofuran (THF) (Merk Millipore), and chloroform (JT Baker) were HPLC grade. Ammonium acetate salt was purchased from Sigma-Aldrich. All other chemicals were at least of analytical grade. Deionized water (18.2 MΩ cm) was generated by a Milli-Q Advantage A10 system and used throughout the experiment.

Stock solutions were prepared in acetonitrile independently at a concentration of 100 mg L^{-1} and serially diluted to the proper concentrations of the spiking solutions (5000, 1000, 500, 200, 10 and 1 μg L^{-1}). All solutions were stored at - 20 ºC.

The lab-made domestic sewage was adapted from Santos et al. (2016), with the following composition (mg L^{-1}): beef extract (260), sucrose (45), soluble starch (142.5), NaCl (500), $MgCl_2.6H_2O$ (14), and $CaCl_2.2H_2O$ (9). A micronutrient solution added to avoid limitations arising from a shortage of micronutrients in anaerobic processes was also in the composition of the medium (Touzel and Albagnac (1983). The anaerobic sludge was collected from an Up-flow Anaerobic Sludge Blank (UASB) reactor treating poultry slaughterhouse wastewater (Avícola Dacar, São Paulo, Brazil). Samples were filtered in combi syringe filters with a coarse glass fiber (GF) pre-filter and a small-pore membrane as the main filter (1.0/0.20 μm).

Instrumentation

TBBPA was identified and quantified using a high-performance liquid chromatography (HPLC Agilent technologies 1260 Infinity) (Palo Alto, CA) coupled with a hybrid triple quadrupole-linear ion trap mass spectrometer ABSciex QTrap® 5500 (AB SCIEX, Foster, CA)

equipped with an ESI source (TurboV™). Chromatographic separation was performed on an InfinityLab Poroshell 120 EC-C18 (3.0 x 50 mm, 2.7 µm) preceded by a guard column (pre-column HPLC, Sb-C18, 20 x 4.6 mm, 1.8 µm) kept at 30 °C and the injection volume was 15 µL. The mobile phase consisted of water (10%) and acetonitrile (90%) in isocratic mode and the pumps were set to a 300 µL min^{-1} flow rate. The mass spectrometer (MS) equipped with an electrospray ionization source was operated in negative-ion mode (-ESI) and the Turbo V™ ion source parameters were optimized by flow injection (FIA) as follows: Curtain gas (CUR) 20 volts, Collision gas (CAD), Source temperature (TEM) 500 ºC, Ion source gas (GS1) 50 psi, Ion source gas (GS2) 40 psi, and Ion spray voltage (IS) -4000 Volts. The declustering potential (DP), collision energy (CE), and cell exit potential (CXP) were optimized by direct infusion of TBBPA and $^{13}C_{12}$-TBBPA for each transition (Table 2.1). The equipment was operated under Selective Reaction Monitoring (SRM) mode with a dwell time of 200 ms. Two MS/MS ion transitions (product ion) were monitored for TBBPA (447.7 and 417.7 *m/z*) and C^{13}-TBBPA (457.8 and 428.7 *m/z*); the most intense transition was used for quantification and the second one was used for confirmation. The initial MS and separation parameters were based on the methods proposed by Saint-Louis and Pelletier (2004) and Liu et al. (2017).

Table 2.1 – SRM parameters for quantitative and qualitative determination of TBBPA and mass-labelled internal standard.

Compound	Precursor ion (Q_1) (m/z)	Product ion (Q_3) (m/z)	DP (Volts)	CE (Volts)	CXP (volts)
TBBPA	543.0	447.7	-190	-42	-11
TBBPA	543.0	417.7	-140	-50	-25
$^{13}C_{12}$-TBBPA	555.0	457.8	-140	-46	-21
$^{13}C_{12}$-TBBPA	555.0	428.7	-140	-54	-17

Chironomus sancticaroli and Daphnia magna culture and sampling

Chironomus sancticaroli and Daphnia magna cultures were maintained at the Aquatic Ecology Environment Laboratory, University of São Paulo. *Chironomus sancticaroli* was cultivated in plastic trays following the recommendations of Dornfeld et al. (2019) and the OECD - Organization for Economic Co-operation and Development (2004). The culture was maintained in dechlorinated tap water with constant aeration, presence of inorganic fine sediment and fed once a week with macerated Tetramin® vitamin and fish food. The organisms were kept in a temperature-controlled room (25 ± 2 ºC) and dark cycle of 12h light:12h dark. *Daphnia magna*

culture was maintained as outlined in the ABNT - Brazilian Association of Technical Standards (2016) and OECD (2004), in a 2 L glass bottle containing reconstituted water, vitamins (VitaChem® and Seachem Prime®) and food (1 $mL.L^{-1}$ of Tetramin® at a concentration of 5g L^{-1} and $3x10^5$ *Raphidocelis subcaptata* cells mL^{-1}). The culture medium was replaced, and the organisms were fed three times a week. Crustaceans were kept in a Solab SL-224 incubator with a dark cycle of 16 h light: 8 h dark and temperature of 19 ± 2 °C.

To compose each sample before spiking them with the correspondent stock solution of TBBPA to final concentrations from 5 to 1000 ng g^{-1}, *Chironomus sancticaroli* organisms (larvae from IV instar) and *Daphnia magna* neonates (less than 24 h of life) were sampled directly from the culture and weighed on an analytical balance until a wet weight of 10±1 mg, which corresponded to about 5 to 8 larvae for *C. sancticaroli* and 60 to 80 for *D. magna* neonates. The organisms were macerated and dried in an oven at 50 °C before contamination.

Solid matrix preparation

An aliquot of the anaerobic sludge was washed in ultrapure water 3 times by vortex homogenization followed by centrifugation in order to wash out any residual soluble organic matter. Each sample had 0.5 g (wet weight, ww) of the centrifuged sludge, which had about 90% of water (dry weight of 50 mg). For the *D. magna* and the *C. sancticaroli* matrices, each sample had 10 mg (ww). Samples were spiked with TBBPA to a final concentration of 5, 50, 100, 250, 500, 750, 1000, and 10.000 ng g^{-1} by adding different solutions in acetonitrile, left at room temperature (25 °C) for 24 h to evaporate the organic solvent, and kept sealed at 3 °C for 24 h to equilibrate. Before the spiking experiment, all samples were ground and dried overnight in an oven at 50 °C to preserve the adsorptive surface.

Chloroform and methanol (1:4, v/v) were used to perform the pre-extraction of the analyte. This mixture was the most appropriate for TBBPA extraction among the non-polar extraction (dichloromethane and chloroform) and dispersive (acetonitrile and methanol) solvents that were tested. 1 mL of the extraction solution was added to the spiked samples and sonicated for 15 minutes at 25 °C. Samples were centrifuged and filtered through syringe filters with a coarse glass fiber (GF) pre-filter and a small-pore membrane as the main filter (1.0 /0.20 μm). This procedure was performed three times. The organic extracts were combined and concentrated to approximately

100 μL and then diluted with ultrapure water to a final volume of 1 mL, which was subjected to the ultrasound dispersive liquid-liquid microextraction (US-DLLME).

Dispersive liquid-liquid microextraction (DLLME)

This technique was applied based on the liquid-liquid extraction optimized by Wang et al. (2013). In this work, sample and dispersive solvent (DS) volumes were reduced by 80% aiming at minimizing the residues generated and the costs incurred by the method without losing its efficiency. Additionally, the time procedure was also reduced (ultrasonication and centrifugation duration). The optimum volumes of dispersive and extraction solvents were evaluated by Analysis of Variance (ANOVA) and the Tukey test to compare if the tested volumes led to significantly different peak areas. Thus, 1 mL of the samples was placed in a 1.5 mL Eppendorf® microtube with 120 μL of THF (DS) and 25 μL of chloroform (Extraction solvent, ES). The mixture was manually shaken for 5 seconds, ultrasonicated for 2 minutes, and centrifuged at 14000 rpm for 1 minute. The sedimentary phase (25 μL) was transferred using an automatic pipette to a *vial* containing 925 μL of acetonitrile and 50 μL of the stable isotope internal standard solution prior to analysis by HPLC/MS-MS. Every sample set included a QA/QC check of a matrix blank, a blank sample (ultrapure water), and three samples containing the magic mix solution (25% Methanol, 25% Acetonitrile, 25% Propanol, and 25% ultrapure water acidified with formic acid, 1%) in LC-MS vials, in a 10% frequency (one quality control run per ten matrix samples).

Method validation

Linearity, limits of detection and quantification, repeatability, precision, accuracy, recovery and storage effect were measured for method validation. The LOD and limit of quantitation (LOQ) were determined by injecting samples to obtain signal-to-noise ratios of 3 and 10 times, respectively. Precision, accuracy, and recovery were assessed according to Matuszewski et al. (2003). Intraday and interday precision (Relative standard deviation, RSD) were determined by the replicate analyses (n = 3) at low, medium, and high concentrations of each calibration curve. The linearity of each standard curve was confirmed by plotting the peak area ratio of the analyte to the internal standard versus TBBPA nominal concentration. The residuals were evaluated for homoscedasticity via the Breusch-Pagan test (Breusch and Pagan, 1979) and autocorrelation via

the Durbin-Watson test (Durbin and Watson, 1951). The analysis of variance, F-tests, and t-test were conducted to verify the linear regression quality and lack-of-fit significance (Snedecor & Cochran, 1989).

The accuracy of the method was expressed by the calculated concentration/spiked concentration ratio percentage. The recovery was determined by comparing the mean peak area ratio of samples in which the analyte was spiked before extraction to the samples that were spiked after extraction (Matuszewski et al., 2003). The matrix effect was not investigated because the slope of the calibration curve obtained using the area ratio analyte signal/internal standard signal versus analyte concentration is independent of the matrix composition, considering that the analyte and the internal standard co-elute (Hewavitharana, 2011).

The storage period test was conducted to evaluate the stability of the extracts for the domestic sewage matrix. Two sets of extracts at low, medium and high concentrations each were analyzed by HPLC-ESI-MS/MS (n=3). One was stored at 3°C and the other at – 20 °C for 10 days. After this period, the vials were ultrasonicated for 10 seconds and re-analyzed.

Real sample analysis

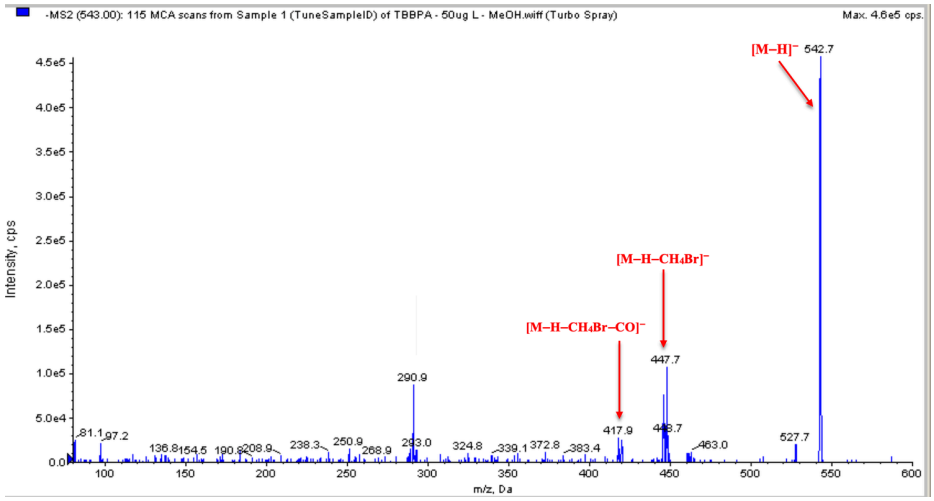
To evaluate the applicability of the proposed method, samples of domestic sewage, anaerobic sludge, and *D. magna* and *C. sancticaroli* were analyzed. For the aquatic organisms, acute toxicity bioassays were carried out in triplicate by exposing 60 neonates of *Daphnia magna* and 6 larvae of *Chironomus sancticaroli* in 250 mL of TBBPA aqueous solution at a concentration of 100 μg L^{-1} for 48 h and 96 h, respectively (unpublished data). After exposure, the organisms were collected, and the proposed method was performed to investigate the bioaccumulation.

The biodegradation of TBBPA by anaerobic digestion was evaluated in domestic sewage (unpublished data). The experiment was conducted in batch reactors maintained in constant agitation at 150 rpm at 30°C for 10 days. TBBPA was added to the medium at a concentration of 100 μg L^{-1}. At the end of the experiment, TBBPA was quantified in both aqueous and solid matrices.

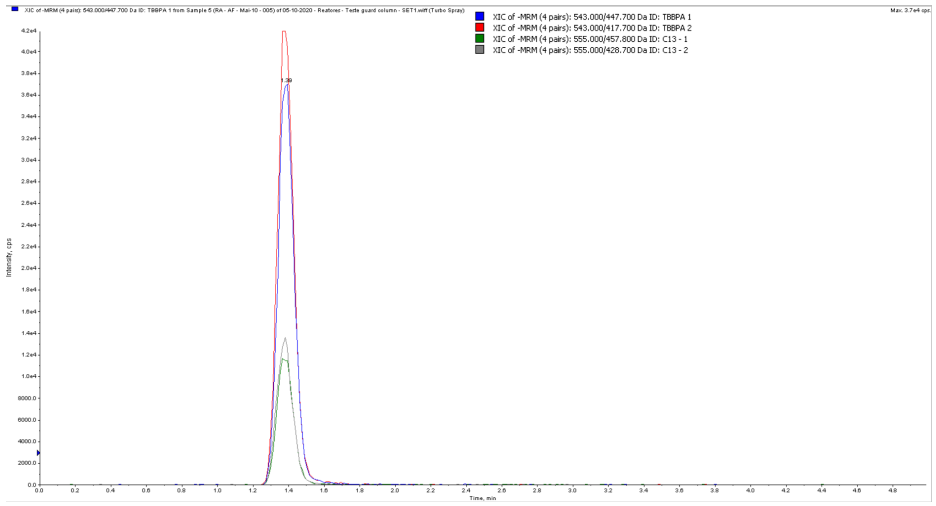
Results And Discussion

Optimizing HPLC conditions and ESI-MS parameters

Different combinations of organic solvents (acetonitrile and methanol) and aqueous solvents (ultrapure water and ammonium acetate 5 mM) were tested, as well as different injection volumes (5, 10, 15 and 20 µL), for optimal separation conditions and analytical efficiency. No gradient elution procedure was necessary and ammonium acetate (5 mM) (A) and acetonitrile (B) (10/90%, v/v) in isocratic mode yielded better results. The column temperature was 30 °C for a total run time of 5 min (TBBPA and $^{13}C_{12}$-TBBPA retention time of 1.4 min). TBBPA was quantified by the determination of product ions in negative-ion mode (ESI-) while the selected reaction monitoring (SRM) mode was carried out for the acquisition. The transitions 417.7 *m/z* [M-H-CH_4Br-CO]$^-$ and 447.7 *m/z* [M-H-CH_4Br]$^-$ were used for quantification and confirmation, respectively. These product ions were selected considering the highest signal obtained in the SRM optimization of transition, chromatogram stability, and previous reports in the literature (Saint-Louis et al., 2004; Liu et al., 2019). Figure 2.1a shows the mass spectra of TBBPA and the possible structures of product ions according to their molecular formulas and the similar fragmentation properties, as previously reported (Liu et al., 2017). Figure 2.1b shows the mass chromatogram of TBBPA and the internal standard under the optimized condition. Further mass chromatograms of TBBPA and $^{13}C_{12}$-TBBPA for each studied matrix are presented in the supplementary data (Appendix A).



(a)

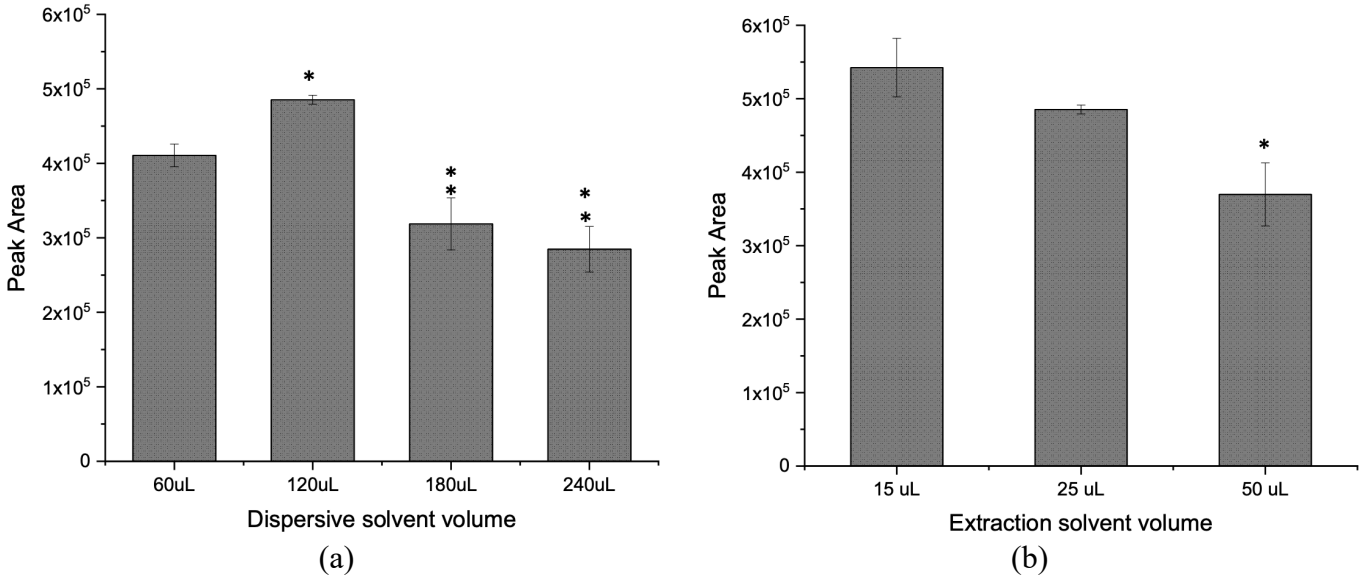


(b)

Figure 2.1 – Mass spectra of TBBPA (a) and mass chromatogram of TBBPA and 13C12-TBBPA in optimized instrumental conditions.

Method performance

Dispersive and extraction solvents volume in the DLLME. To evaluate the ideal dispersive solvent (DS) volume, samples were prepared (n=3) with 25 µL of extraction solvent (ES), which were optimized by Wang et al. (2013), testing the volumes of 60, 120, 180, and 240 µL. The tested DS volumes led to statistically different peak areas (ANOVA p-value of 3.41E-5). The Tukey test showed that 60 µL of DS led to a TBBPA peak area which was statistically different from the results obtained with 120, 180, and 240 µL (p-values of 0.0247, 0.0079 and 0.0010, respectively). However, the greatest recovery of TBBPA (highest signal intensity) was obtained using 120 µL of DS (Figure 2.2a). Afterward, different volumes of the ES were tested (15, 25, and 50 µL) (n=3) (Figure 2.2b) and led to statistically different results (ANOVA p-value of 0.0022). It was observed that the average peak areas obtained using 50 µL of ES was statistically different from the averages obtained using 15 and 25 µL by Tukey test analysis. Even though 15 µL led to the analyte's highest signal intensity, it was difficult to accurately remove the sedimentary phase from the bottom of the centrifuged microtube. For this reason, 25 µL was used in the proposed method.



*, ** presence of statistical difference ($p < 0.05$).

Figure 2. 2 – Average TBBPA peak area and respective standard deviation obtained for different volumes of dispersive and extraction solvent in the DLLME method.

Merit figures for method validation. Linear regression analysis was carried out by plotting the peak area ratio (peak area of the analyte divided by the IS peak area) versus analyte concentration, using five levels in triplicate experiments. The linear range was determined based on the environmental occurrence of TBBPA in each studied matrix and is presented in Table 2.2.

Table 2.2 – Weighted linear regression coefficients and Durbin-Watson test values for residuals analysis.

Matrix	Weighting factor	R	R^2	Adjusted R^2	Std. Error of the Estimate	Durbin-Watson
DS (1 to 120 μg L^{-1})	1/x	.998	0.996	0.996	0.177	2.1
AS (0.05 to 1 μg $gSSV^{-1}$)	1/x	.985	0.971	0.969	0.005	1.3
Dm (0.1 to 10 μg g^{-1})	1/x	.999	0.998	0.998	0.001	1.4
Cs (0.1 to 10 μg g^{-1})	Ln(x)	.986	0.973	0.971	0.159	2.4

The values of correlation (R) and regression coefficients (R^2) were considered adequate, demonstrating good linearity for the studied intervals. The residuals of the linear regression were examined for obvious patterns and homoscedasticity was confirmed by the Breusch-Pagan test (Breusch and Pagan, 1979) for DS and AS matrix only, in which the residual variability across all concentration levels was constant ($p > 0.05$). For the Dm and Cs matrices, Breusch-Pagan statistics were significant (p-value of 0.001 and 0.004, respectively). Heteroscedasticity is commonly related to a wide linear range (0.05 to 10 μg^{-1}) and performing weighted linear regression is an appropriate way to better adjust heteroscedastic data (Deng et al., 2016; Valese et al., 2016). The weighting

factor chosen for each matrix (Table 2.2) yielded the smaller relative error among the empirical weighting factors mostly used. Even though homoscedasticity was observed in the DS and AS matrices, weighted linear regression was applied to enhance accuracy.

Durbin-Watson (DW) test (Table 2.2) was performed for evaluating the autocorrelation of residuals. The DW-values ranging from 1.3 to 2.4 demonstrates that residuals are uncorrelated (Durbin and Watson, 1951; Pastor-Belda et al., 2018).

Table 2.3 presents the ANOVA statistics for the weighted linear regressions, in which the F-test was performed to evaluate the quality of the regression for each matrix. For each curve, highly significant regression ($p < 0.01$) was obtained. The lack of fit was also evaluated and was non-significant ($F_{exp} < F_{2,10}$) for a significance level of 95%.

Table 2.3 – ANOVA statistics for linear regression of calibration curves.

		SS	Df	MS	F	p-value
DS	Regression	97.37	1	97.379	3099.97	7.45E-17
	Residual	0.41	13	0.031		
	Total	97.79	14			
AS	Regression	9.97E-03	1	9.97E-03	433.38	2.29E-11
	Residual	2.99E-04	13	2.30E-05		
	Total	1.03E-02	14			
Dm	Regression	7.46E-03	1	7.00E-03	5898.87	1.15E-18
	Residual	1.60E-05	13	0.00E+00		
	Total	7.48E-03	14			
Cs	Regression	1.17E+01	1	1.17E+01	463.55	1.50E-11
	Residual	3.29E-01	13	2.50E-02		
	Total	1.21E+01	14			

Table 2.4 presents the coefficients that describe the calibration equations for all matrices. The slope coefficients are statistically significant ($p < 0.01$) by the t-test performed at the 99% significance level.

Table 2.4 – Calibration curves parameters and t-test values for slope significance.

Matrix		Coefficients		t-test	p-value
		B	Std. Error		
DS	(Constant)	-1.14E-01	7.20E-02	-1.58	1.37E-01
	Slope	5.95E-02	1.00E-03	55.68	7.45E-17
AS	(Constant)	-5.29E-02	1.79E-02	-2.95	1.12E-02
	Sloe	1.65E-03	7.90E-05	20.82	2.29E-11
Dm	(Constant)	-1.32E-02	3.58E-03	-3.70	2.67E-03
	Slope	4.82E-04	6.00E-06	76.80	1.15E-18
Cs	(Constant)	1.36E-02	2.10E-02	0.655	0.523991
	Slope	8.20E-05	0.00E+00	21.53	1.50E-11

Limits of Detection (LOD), Limits of Quantification (LOQ), accuracy, intraday and interday relative standard deviation (RSD), and method recovery are presented in Table 2.5 for low (L), medium (M), and high (H) concentrations within the calibration curve of each matrix. These parameters ranged in agreement with international acceptance criteria (Munch, 2005).

Table 2.5 – Performance of the US-DLLME-LC-MS method for different matrices

Matrix	LOD	LOQ	Accuracy (%)	RSD intraday (%)	Repeatability, RSD interday (%)	Recovery (%)
DS	2.0 ng L^{-1}	1 µg L^{-1}	L: 113.9	L: 4.1	L: 8.2	L: 50.7
			M: 95.2	M: 6.6	M: 7.7	M: 48.5
			H: 106.3	H:2.6	H: 3.9	H: 48.7
AS	2.0 ng $SSVg^{-1}$	0.1 µg $SSVg^{-1}$	L: 98.3	L: 3.1	L: 11.9	L: 32.5
			M: 89.5	M: 9.6	M: 8.7	M: 30.9
			H: 108.0	H: 6.2	H: 5.8	H: 35.5
Dm	2.5 ng g^{-1}	0.05 µg g^{-1}	L: 107.6	L: 3.9	L: 8.1	L: 36.5
			M: 98.6	M: 4.6	M: 5.7	M: 43.5
			H: 100.3	H: 5.0	H: 8.7	H: 42.9
Cs	5 ng g^{-1}	0.05 µg g^{-1}	L: 127.9	L: 3.1	L: -	L: -
			M: 99.5	M: 16.2	M: 9.6	M: 13.6
			H: 97.4	H: 8.5	H: -	H: -

Low (L), medium (M), and high (H) concentrations for each matrix: DS (1, 40, and 120 µg L^{-1}), AS (0.1, 0.25, and 1 µg $SSVg^{-1}$), Dm (0.1, 1, and 10 µg g^{-1}), Cs (0.1, 0.5, 10 µg g^{-1}).

For the storage period test performed for DS extracts, the average intraday repeatability was 14.9 and 4.2% for the sets stored at 3°C and – 20 °C for 10 days, respectively, demonstrating good stability of the extracts stored at – 20 °C. Accuracy and RSD greater than 120%, and 15%, respectively, were obtained for the *C. sancticaroli* matrix, which may be related to the complexity of the matrix and wide linearity range. Additionally, these results are comparable to RSD (%) and correlation coefficients reported for a MSPE-HPLC-UV method to determine TBBPA in fish samples (Hu et al., 2019) and a SFME-PS-MS method for analyzing PFOS and PFOA in the body fluid of *Daphnia magna* (Deng et al., 2016). LOD of 0.002 µg L^{-1} for the sewage sludge matrix is at least 40-fold smaller compared to results reported for liquid samples of lower complexity, demonstrating the method's sensitivity (Wang et al., 2013; Zhang et al., 2016; Liu et al., 2017). For the other matrices, the LODs obtained in this study are comparable to other limits reported for a DLLME-GC-MS method in environmental matrices (Erarpat et al., 2019).

The method recovery (Table 2.5) for each matrix was relatively lower than the method recovery (%) reported in the literature. Greater recovery (84.2 - 88.6%) was obtained by Erarpat et al. (2019) possibly due to the derivatization process and the greater sample volumes reported by

these authors to determine organotin compounds in fish and mussel samples. Generally, extraction methods for environmental matrices such as wastewaters, sludge, animal tissues, and aquatic organisms require more robust and expensive techniques, using greater volumes of organic solvents and with longer sample preparation time (Zhang et al., 2016; Kotthoff et al., 2017; Liu et al., 2017). Most environmental samples are pretreated or extracted with conventional procedures such as SPE or LPE, leading to great results (Saint-louis et al., 2004; Kabir et al., 2017; Burato et al., 2020). It should be noted that the costs and waste generated by the methods described above are the real limitations of these processes.

Additionally, sample digestion is not required in the presented method, reducing the time required to process samples and technique complexity. Table 2.6 presents a more detailed description of published studies regarding the use of liquid-liquid and solid-phase based extraction methods in environmental samples, especially for TBBPA and related flame retardants, with equivalent LOD and LOQ results obtained in this work.

Table 2.6 – Latest extraction methods for determining TBBPA and other flame retardants on environmental matrices.

Analyte	Samples		Technique	Solvent volume per sample		Extraction time (min)**	LOQ	LOD	Authors
	Nature	Amount		Dispersive	Extraction				
Butyltin compounds (MBT, DBT, TBT)[a]	Fish and mussel	8 mL	DLLME-GC-MS	1.64 mL	300 uL	30	1.3 - 2.1 0.4 - 0.6 μg kg^{-1}	0.4 - 0.6 μg kg^{-1}	Erarpat et al. (2019)
TBBPA, BPA and OP	Urine	10 mL	LLE-LC-MS	32 mL	8 mL	36	0.05 - 0.25 μg L^{-1}	0.01 - 0.08 μg L^{-1}	Zhang et al. (2016)
TBBPA	Fish	-	MSPE-HPLC-UV	N/A	15 mL*	65	50 ng g^{-1}	15.2 ng g^{-1}	Hu et al. (2019)
PFOS and PFOA	Body fluid of D. magna	10 uL	SFME-PS-MS	N/A	N/A	N/A	0.13 - 0.3 μg L^{-1}	0.04 - 0.09 μg L^{-1}	Deng et al. (2016)
TBBPA/S	Water and sea water	200 mL	LLE-HPLC-MS/MS	N/A	300 mL*	N/A	5 μg L^{-1}	0.12 - 0.19 μg L^{-1}	Liu et al. (2017)
TBBPA and derivatives	urine and fruit juice	5 mL	DLLME-HPLC-UV	600 uL	25 μL	7	0.43 - 2.1μg L^{-1}	0.13 - 0.63 μg L^{-1}	Wang et al. 2013
TBBPA	Soil	1000 mg	HTDMAE-LLE-LC-MS/MS	N/A	12.5 mL*	20	4.6 ng g^{-1}	1.4 ng g^{-1}	Zhao et al. (2016)
Bisphenols	Beverages	10 mL	SPE-LC-MS/MS	N/A	30 mL*	75	5.2 - 75.1 ng L^{-1}	1.6 - 17.6 ng L^{-1}	Regueiro and Wenzl (2015)
TBBPA, HBCD and BDE	Human serum	1 mL	QuEChERS-GC/LC-MS/MS	N/A	18 mL*	12	0.25 - 5 μg L^{-1}	4 - 19 ng L^{-1}	Li et al. (2017)
Bisphenol A	Food items	5000 mg	SPE-GC-MS/MS	N/A	80 mL*	N/A	0.03 μg kg^{-1}	0.1 μg kg^{-1}	Deceuninck et al. (2014)
Bromophenols	Water	100 to 300 mL	SPE-HPLC-MS/MS	N/A	12 mL*	20	-	0.1 - 21.9 ng L^{-1}	Chi et al. (2017)
PeBP, TCBPA and TBBPA	Seafood	500 mg	VH-MSPD-GC-MS/MS	N/A	12 mL*	N/A	0.5 ng g^{-1}	0.2 ng g^{-1}	Chen et al. (2016)

[a]Butylin compounds are not flame retardants. *Methods based on solid-phase extraction;**Time of main extraction procedure described; DLLME: Dispersive Liquid Liquid Microextraction; LLE: Liquid Liquid Extraction; MSPE: Magnetic Solid Phase Extraction; SFME: Slug-Flow Microextraction; HTDMAE: High-Throughput Dynamic Microwave-Assisted Extraction; QuEChERS: Quick Easy Cheap Effective Rugged Safe extraction; VH-MSPD: Vortex-Homogenized Matrix Solid Phase Dispersion.

Compared to the proposed method, even the miniaturized extraction techniques require a greater solvent volume and procedure time (Erarpart et al., 2019; Wang et al., 2013) and/or a more complex and time-consuming apparatus (Deng et al., 2016; Zhao et al., 2016). The results obtained from this study suggest that the sample preparation before the DLLME procedure and the miniaturization of what was proposed by Wang et al. (2013) led to satisfactory results on TBBPA quantification even in complex matrices. For solid environmental matrices, most of the methods rely on solid-phase extraction, with conventional (C_{18} cartridges) (Deceuninck et al. 2014; Regueiro and Wenzl, 2015; Li et al., 2017; Chi et al., 2017) or alternative sorbent materials (Chen et al., 2016; Hu et al., 2019), requiring a large volume of solvents for cartridge activation and analyte elution.

Real sample analysis

To evaluate the applicability of the proposed method, samples of the cited domestic sewage, anaerobic sludge, *D. magna* and *C. sancticaroli* were analyzed. In the anaerobic batch reactor, the final concentration of the contaminant in the domestic sewage and in anaerobic sludge was 0.2 ± 0.03 μg L^{-1} and 507± 79 ng g^{-1}, respectively. For the aquatic organisms subjected to acute toxicity bioassays, TBBPA accumulated at 3.74 μg g^{-1} and 8.87 μg g^{-1} in *D. magna* (48 h exposure) and *C. sancticaroli* (96 h exposure), respectively, indicated that TBBPA is highly bioaccumulative. Generally, Tetrabromobisphenol A bioaccumulation is investigated in fish and fish tissues, ranging from 0.01 to 2.85 ng g^{-1} (ww) (Ashizuka et al., 2008; Tang et al., 2015). Quantifying this micropollutant in *D. magna* and *C. sancticaroli* is highly relevant as they are primary consumers in aquatic ecosystems and are used as prey for high-level consumers, allowing earlier detection of environmental contamination.

Conclusion

This work contributes to the need to develop eco-friendly and miniaturized analytical methods to determine TBBPA in environmental samples. Contrary to what has been used to detect micropollutants in complex matrices, the proposed US-DLLME-LC-MS/MS method allows for a simple, fast, accurate, sensitive and cost-effective quantification procedure. The presented method was able to successfully quantify TBBPA in domestic sewage and anaerobic sludge, which are

matrices that indicate the environmental occurrence of hazardous chemicals. Additionally, it is a pioneer in determining the bioaccumulation of TBBPA in the experimental species *D. magna* and *C. sancticaroli* used for ecotoxicological assessment, suggesting its feasibility for environmental monitoring.

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Chapter 3

Tetrabromobisphenol A (TBBPA) anaerobic biodegradation occurs during acidogenesis

Abstract. This is the first study to bring evidence on the anaerobic biodegradation of TBBPA occurring during acidogenesis in domestic sewage at environmentally relevant concentrations by complex microbial communities. This was accomplished by continuously operating two anaerobic structured bed reactors (ASTBR) for over 100 days under acidogenic (Acidogenic Reactor, AR) and multistep methanogenic (Methanogenic Reactor, MR) conditions. In the AR, the temporal carbohydrates consumption and the acetic acid production were strongly correlated with TBBPA removal by the Pearson's test. The spatial concentration of TBBPA and carbohydrates along the MR and the kinetic degradation profiles corroborate the AR results. It is hypothesized that TBBPA biodegradation in the studied conditions occurs during acidogenesis via the cometabolism supported by non-specific enzymes and the metabolism (dehalorespiration) established by electrons donors such as H_2, which are both produced during the macrocomponents breakdown in the early stages of the anaerobic digestion. The TBBPA mass balance showed that approximately 86.8 ± 0.05 % and 97 ± 0.01 % of the removed TBBPA was biodegraded in the AR and MR, respectively. Furthermore, TBBPA biodegradation went further than reductive debromination as total phenols were detected in the reactors' effluent.

Introduction

Tetrabromobisphenol A (TBBPA) is the most common flame retardant applied to electric and electronic equipment, epoxy resins, and plastic products to meet fire safety requirements and has been detected in air, dust, sediment, biota, and water. In water bodies, its concentrations range from few picograms per liter to 4870 ng L^{-1} (Yang et al., 2012; Wang et al., 2014; Zhou et al., 2014; Qu et al., 2016; Daso et al., 2017; Rothenbacher and Pecquet, 2018) and in sewage sludge samples, from undetectable to 1329 ng g^{-1} dw (dry weight) (Gorga et al., 2013). In a study conducted in South Korea, TBBPA was the predominant brominated flame retardant (BFR) detected and quantified in both influent and effluent of various wastewater treatment plants in concentrations ranging from 122 to 13144 pg L^{-1} (Kim et al., 2016). Concerns on TBBPA environmental

occurrence led to studies on technologies capable of converting this chemical into less toxic and less persistent compounds. TBBPA is immunotoxic, neurotoxic, and may cause endocrine disruptions in organisms. It is also associated with the development of cancer in the uterus of rats, maternal transmission between fish generations, and genetic mutation in frogs (Veldhoen et al., 2006; Covaci et al., 2009; Nyholm et al., 2008; Yang et al., 2012; NTA, 2014). The biological degradation of halogenated micropollutants is assumed to be possible under all redox conditions by a variety of bacterial and archeal species (Hardman et al., 1991; Peijnenburg et al., 1992; Häggblom et al., 1992; Fetzner , 1998; Ghattas et al., 2017). However, little is known about the microbial population driving flame retardants biodegradation, their symbiosis, syntrophic relationships, and how microbial diversity affects the biological system efficiency. Knowledge on the microbial group actively biodegrading these micropollutants would be the first step to apply biochemical knowledge on industrial processes.

TBBPA and most of the organic micropollutants (OMP) may be biodegraded via metabolic and cometabolic processes. In the process known as metabolism, the microorganisms use the organic substances to support growth, obtain energy, and to induce enzymes for their assimilation. However, OMPs in wastewaters are usually in concentrations that do not allow metabolic degradation (ng to μg L^{-1}) and an external carbon/energy source is needed to support biomass growth and induce the enzymatic machinery for the biodegradation to occur, which is known as cometabolism (Fischer et al., 2014; Ghattas et al., 2017). Besides the cometabolic-ATP-dependent reductive debromination, dehalogenation may be accomplished by a metabolic mechanism known as dehalorespiration. In this respiratory process, an electron donor such as H_2 would be used as energy source and TBBPA would be used as the electron acceptor in an exergonic dehalogenation reaction, yielding energy that could be used for bacterial benefit (Fetzner, 1998; El Fantroussi et al., 1998; Zhang et al., 2005).

The anaerobic degradation of TBBPA in bioreactors has been mostly investigated by evaluating operational parameters (pH, temperature, concentration, carbon source, and others amendments) and understanding isolated bacterial strains functions (Ronen et al., 2000; Iasur-Kruh et al., 2010; Chang et al., 2012; Peng et al., 2013; Yang et al., 2016; Peng et al., 2017; Fan et al., 2017; Islam et al., 2018; Cheng et al., 2019; Lefevre et al., 2019; Lin et al., 2020). However, the role of each anaerobic digestion stage on TBBPA biodegradation has not been addressed. Additionally, most of the studies regarding TBBPA degradation in biological systems were

conducted at TBBPA concentrations surpassing by several orders of magnitude its typical environmental levels. This aspect may lead to metabolic pathways and outcomes not necessarily transferable to environmental settings by favoring metabolic routes in which TBBPA supports bacterial growth.

The results published by previous investigations (Ronen et al., 2000; Arbeli and Ronen, 2003; Arbeli et al., 2006; Iasur-Kruh et al., 2010) state the role of intermediate products generated in the methanogenic process on TBBPA bioconversion. The authors observed that when archaeal species were chemically inhibited (by 2-bromoethanesulfonate, BES), TBBPA debromination was delayed and when bacterial species were inhibited (by ampicillin), the degradation did not occur at all. In sequence, the effect of different electron donors (ethanol, lactate, acetate, and butyrate) suggested that best results on TBBPA conversion (initial concentration of 50 mg L^{-1}) were associated with carbon sources yielding H_2 production (ethanol>lactate>butyrate), while no conversion was observed when acetate was used. Even though the applied concentration of TBBPA could support other metabolic routes using the micropollutant as carbon and energy source for microbial growth, this study pioneeringly suggested that debromination as a respiratory process (Fetzner, 1998), in which TBBPA acts as electron acceptor and H_2 as electron donor, prevails on anaerobic TBBPA degradation. For the authors, the process driving TBBPA biodegradation was induced by the H_2 produced in the acidogenesis of external carbon sources, as the substrate that could not yield H_2 production (acetate) did not support the degradation of TBBPA, despite supporting biomass growth and maintenance. Moreover, the role of H_2 as the energy source on TBBPA biodegradation has been stated in more recent studies (Wei et al., 2018; Lin et al., 2020) and the use of acetate was further confirmed as not the most suitable amendment to improve the TBBPA bioconversion efficiency (Lefevre et al., 2016).

The beneficial effect of amendments yielding H_2 production on TBBPA degradation leads to the idea of the metabolic dehalorespiration being the main mechanism of TBBPA debromination. However, these amendments also enable TBBPA cometabolic reactions driven by non-specific enzymes produced to degrade macrocomponents (i. e. organic matter). Most importantly, both the dehalorespiration and the cometabolic degradation are favored during acidogenesis. Even though these findings (Iasur-Kruh et al., 2010; Lefevre et al., 2016; Wei et al., 2018; Lin et al., 2020) suggest that TBBPA bioconversion occurs during acidogenesis, there are no studies directly

relating these processes nor further investigations on TBBPA anaerobic degradation in an acidogenic medium.

Therefore, this study aimed at uncovering which anaerobic digestion stage drives TBBPA degradation at environmentally relevant concentrations by complex microbial communities. To this objective, two anaerobic structured bed reactors (ASTBR) were operated continuously for over 100 days under acidogenic (Acidogenic Reactor, AR) and under multistep methanogenic (Methanogenic Reactor, MR) conditions to evaluate if the TBBPA bioconversion occurs during acidogenesis or if the subsequent anaerobic digestion stages play an important role on the breakdown of this micropollutant.

Material And Methods

Chemicals and materials

Tetrabromobisphenol A (TBBPA, 4,40-Isopropylidenebis (2,6-dibromophenol), 97% purity, CAS 79-94-7) and the stable isotope-labeled internal standard ($^{13}C_{12}$-TBBPA) were purchased from Sigma-Aldrich (Brazil) and from Wellington Laboratories (Guelph, Canada), respectively. All other chemicals used in the lab-made domestic sewage composition, macronutrients, and micronutrients were at least of analytical grade. Samples were filtered in combi syringe filters with a coarse glass fiber (GF) pre-filter and a small-pore membrane (PTFE) as the main filter (1.0/0.20 μm).

Bioreactors configuration and operational conditions

Two bench-scale anaerobic structured-bed reactors (ASTBR) were used with polyurethane foam as support material for biomass adhesion. This reactor configuration and support material have been extensively applied in anaerobic processes for macro and micropollutants removal (Oliveira et al., 2017; Carneiro et al., 2019; Macêdo et al., 2019). The schematic description of the ASTBR is presented in the Figure B.1 in the supporting information (Appendix B). Both reactors were made of acrylic with a working volume of 1.5 L, length of 65 cm, and internal diameter of 8 cm. The ASTBRs bed consisted of six vertical strips of polyurethane foam with 1 cm^2 of base and 35 cm of length, with porosity of 92%, apparent density of 23 g L^{-1}, and specific surface area of 43.8 m^2 g^{-1} (Silva et al., 2006).

The inoculum used in the continuous reactors was anaerobic sludge collected from an Up-flow Anaerobic Sludge Blanket (UASB) reactor treating poultry slaughterhouse wastewater (Avícola Dacar, São Paulo, Brazil). The inoculum was pretreated in the acidogenic reactor by the acid shock method (Penteado et al., 2013). In brief, the pH of the sludge was adjusted to 3.0 using HCl (1 mol L^{-1}) and maintained at 30°C for 24 hours. Then, the system was started and operated in the chosen hydraulic retention time (HRT) of 1.2 h to favor acidogenic conditions. The acidogenic reactor (AR) and the methanogenic reactor (MR) were fed with lab-made wastewater made to simulate the soluble fraction of domestic sewage with a theoretical influent chemical oxygen demand (COD) of 500 mgO_2 L^{-1}. The lab-made domestic sewage was adapted from previous literature (Santos et al., 2016) and a detailed description of the substrate feed, macro, and micronutrients added to the reactors can be found in the SI. The HRT was set at 1.2 h and 6 h for the AR and MR, respectively. Both reactors were maintained in a temperature-controlled chamber at 30 °C.

The stock solution of TBBPA was prepared in methanol and stored in a freezer (−20 °C). TBBPA was added to a final concentration of 50 and 100 µg L^{-1} in the AR and MR feed solutions, respectively, throughout the operational period (100 days). The influent TBBPA concentrations differed in each reactor due to the influent pH of the domestic sewage (pH of 6.92 ± 0.13 and 7.95 ± 0.12, for AR and MR, respectively), which is directly related to the TBBPA chemical speciation and solubility. The TBBPA pK_{a1} and pK_{a2} values are 7.5 and 8.5, respectively, meaning that the ionized forms of TBBPA will become prevalent at pHs > 7-8 and that at lower pHs TBBPA will be present essentially in the undissociated form (Bureau, 2006). Therefore, 100 µg L^{-1} of TBBPA at the pH value of AR influent was not possible to be consistently maintained. Based on TBBPA concentrations in surface waters (Yang et al., 2012), the studied concentrations simulated contaminated effluents with a concentration approximated to relevant environmental settings.

Bioreactors monitoring and analytical methods

The bioreactors performance was monitored by physicochemical analyses and TBBPA monitoring in the liquid and solid matrices. Carbohydrate concentrations were determined using the colorimetric method (DuBois et al., 1956). The suspended solids (2540-E), chemical oxygen demand (COD) (5220-D), and pH (4500-H+ B) were measured in accordance with the Standard Methods (APHA, 2017). Phenol was measured by the modified spectrophotometric method for the

determination of trace amounts of phenol in water (Kang et al., 2000) with the calibration curve ranging from 5 to 500 μg L^{-1}. The biogas composition (hydrogen, methane, and carbon dioxide) was determined with a gas chromatograph (GC-2010, Shimadzu®) equipped with a thermal conductivity detector with argon as the carrier gas and a CARBOXEN® 1010 Plot (30 m, 0.53 mm) column (Penteado et al., 2013). The biogas production flowrate was measured using a semi-continuous gas meter with a J-tube hydraulic valve (Veiga et al., 1990). The data was continuously acquired using an in-house developed software.

The analysis of organic acids (OA: lactic, formic, acetic, propionic, butyric, isobutyric, valeric, and isovaleric) and alcohols (ethanol and methanol) was performed by gas chromatography using a flame ionization detector (GC-FID, Shimadzu®) according to Adorno et al. (2014) and by a high-performance liquid chromatograph (HPLC, Shimadzu®) equipped with an UV-diode array detector (SDP-M10 AVP), refraction index detector (RID-10A), and an Aminex HPX-87H column (300 mm, 7.8 mm, BioRad®) (Penteado et al., 2013). The limits of detection (LOD) and quantification (LOQ) for organic acids in these methods vary from 0.17 to 12.77 mg L^{-1} and from 0.46 to 38.7 mg L^{-1}, respectively.

TBBPA quantification in the liquid (influent and effluent) and solid (suspended and adhered biomass) matrices was performed by liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis with LOD and LOQ of 2 ng L^{-1} and 1 μg L^{-1}, respectively. The samples were prepared using an ultrasound-assisted dispersive liquid-liquid microextraction procedure (Macêdo et al., 2020). Chromatographic separation was obtained using an Agilent Technologies 1260 Infinity chromatograph equipped with an Agilent Technologies Poroshell 120 EC-C18 column (50 mm × 3.0 mm; 2.7 μm). Tandem mass spectrometric analysis was performed using a 5500 QTRAP hybrid mass spectrometer (ABSciex) equipped with a TurboV™ ion source, operated in the negative electrospray mode (ESI-). Details on HPLC conditions, ESI-MS parameters, and the microextraction procedure are described elsewhere (Macêdo et al., 2020). TBBPA quantification in the solid matrix (adhered biomass) was performed in samples withdrawn from three different sections of the reactors bed for sorption quantification purposes. These sections are indicated in Figure S1 (SI).

Degradation kinetics and statistical analysis

In order to evaluate the degradation kinetics of TBBPA in the continuous reactors, spatial profiles were obtained by collecting samples along the reactors' length. In these samples, TBBPA and carbohydrates concentrations were quantified as well as the volatile suspended solids. A first-order kinetic model with a residual concentration (Camargo et al., 2002) was adjusted to the experimental data. In which C is the concentration in the bulk, C_0 is the concentration in the influent, θ_h is the HRT, and C_R is the residual concentration when the reaction rate value is zero. The apparent first-order kinetic is expressed by the k_{app} constant. Considering that the reactors' flow-pattern as plug flow, as previously demonstrated (Carneiro et al., 2019), samples collected in the points along the reactor length (Figure S1) represented different HRTs, from 0 to 1.2 h (AR) and from 0 to 6 h (MR).

$$C = C_R + (C_0 - C_R)e^{-k^{app}\,\theta} \text{ (Equation 1)}$$

All the statistical analyses (ANOVA, Pearson's correlation matrix, and first-order kinetic model fitting) were performed using the IBM software SPSS® and Rstudio version 1.2.5001.

Results And Discussion

Bioreactors' performance: TBBPA and organic matter biodegradation

Table 3.1 presents the overall removal and biodegradation efficiencies in the AR and MR. Data is presented and discussed considering the period in which the reactors reached a pseudo-steady-state (from day 50 to day 100), with the removal efficiencies varying less 15%.

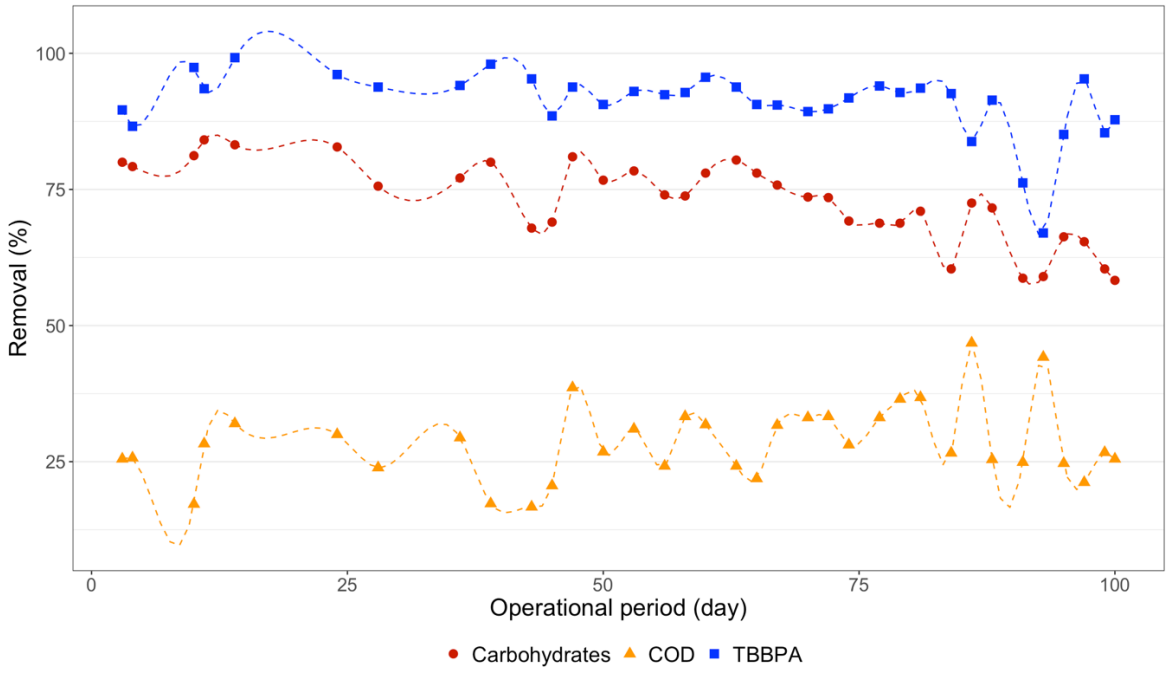
Table 3.1 – Removal and bioconversion of TBBPA and organic matter in the acidogenic (AR) and methanogenic (MR) reactors.

		AR From day 50 to 100	MR From day 50 to 100
TBBPA	Influent ($\mu g \cdot L^{-1}$)	43.7± 5.1	89.6 ± 10.2
	Effluent ($\mu g \cdot L^{-1}$)	4.5 ± 2.7	1.7 ± 0.9
	Removed (%)	89.4 ± 6.5	98.0 ± 1.2
	Bioconverted (%)	86.8 %	97%
	Adsorbed (%)	≅ 3%	≅ 1%
Organic Matter	Carbohydrates removal (%)	72.9 ± 7.5	≅ 100 *
	COD removal (%)	28.5 ± 6.9	90.9 ± 3.4

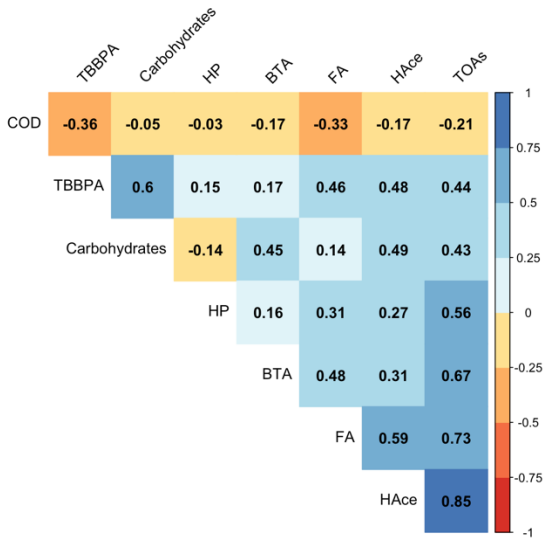
*The carbohydrates concentrations in the effluent were below the detection limit (5 $mg \cdot L^{-1}$).

The volume of biogas produced per day in the AR and MR was 97 ± 83 and 680 ± 118 mL, respectively. H_2, N_2, CH_4, and CO_2 gases were detected in the AR biogas throughout the operational period while no H_2 was detected in the MR biogas. Even though CH_4 accounted for most of the biogas content (68.8 ± 22.9 % for the AR and 93.7 ± 4.7 % for the MR) in both reactors, its production in the AR did not affect its acidogenic condition as it represented only 1.1 ± 0.2 % of the influent COD. The pre-treatment to inhibit *Archaeal* activity was still efficient considering the inoculum microbial diversity (Delforno et al., 2017) from a reactor with long-term operation under methanogenic conditions. In addition, the low COD removal efficiency (28.5 ± 6.9%) and the accumulation of organic acids in the AR effluent, which corresponded to 70.5 ± 6.7% of the effluent COD, reassures its predominantly acidogenic condition.

Figure 3.1a shows the temporal profiles of TBBPA, carbohydrates, and COD removal in the AR, which suggest a correlation of TBBPA removal with carbohydrates consumption. Figure 3.1b shows the Pearson's correlation matrix of the main organic acids (OAs) and TBBPA, carbohydrates, and COD removal in the AR. The TBBPA and carbohydrates removal efficiencies in the MR are not displayed graphically because they were all greater than 90% throughout the operational period, leading to low residual organic acids concentrations in the effluent with the predominance of acetic acid in concentrations no greater than 10 mg L^{-1}. In the AR, the acetic acid (73 ± 16.9 mg L^{-1}) was the predominant soluble metabolite produced, followed by propionic (28.6 ± 8.8 mg L^{-1}), butyric (27.8 ± 11.3 mg L^{-1}), and formic (12.2 ± 3.2 mg L^{-1}) acids, corresponding to 71.1 ± 9.0 % of the effluent COD.



(a)



(b)

Figure 3.1 – Temporal TBBPA, carbohydrates, and COD removal efficiencies (a) and Pearson's correlation matrix for the AR (b). The dashed lines are included as a guide to the eyes. TBBPA, carbohydrates and COD objects represent their removal efficiencies. HP: Propionic acid; BTA: Butyric acid; FA: Formic acid; HAce: Acetic acid; TOAs: Total organic acids.

The correlations of TBBPA and organic matter removal in both reactors are graphically expressed in Figure 3.2a and b.

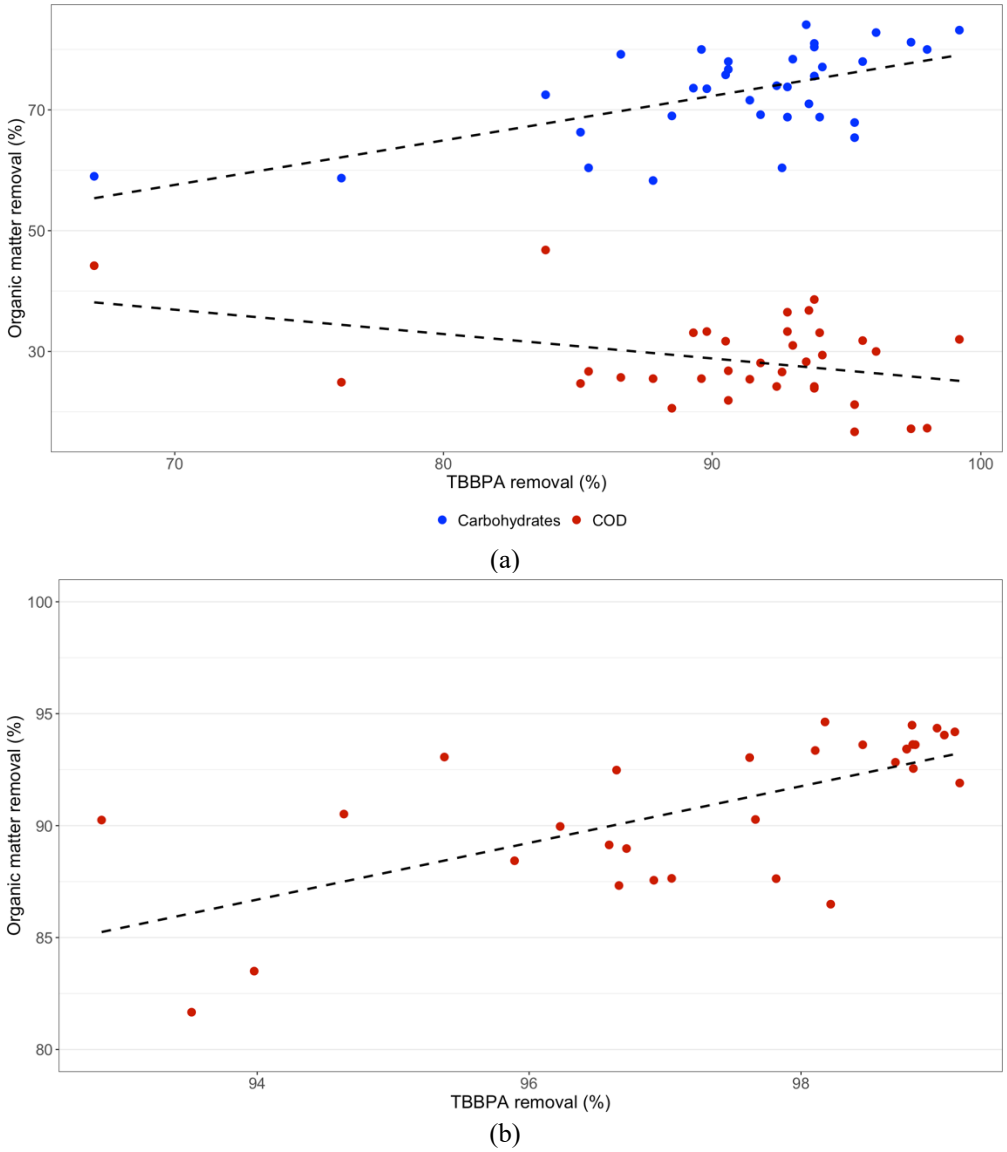


Figure 3.2 – Pearson's correlation of TBBPA removal and organic matter removal in the acidogenic (a) and methanogenic (b) reactors. The dashed lines represent a linear model fitting.

In the AR (Fig. 2a), the Pearson's coefficient strongly and positively correlates carbohydrates consumption (0.600 with p-value < 0.01) and acetic acid production (0.481 with p-value <0.05) with TBBPA removal, suggesting that TBBPA bioconversion occurs during acidogenesis. Additionally, the OA production being also positively (0.442 with p-value < 0.05) (Fig. 1b) correlated with TBBPA biodegradation suggests that the soluble metabolites quantified in the bulk were not used as carbon or energy source in the cometabolic process of TBBPA breakdown.

In the MR, the Pearson's correlation test was also applied and the positive correlation of COD removal with TBBPA biodegradation (Pearson's coefficient of 0.540 with p-value <0.01) (Fig 2b) may raise questions on the strict relationship of carbohydrates consumption vs TBBPA bioconversion. One may conclude that the TBBPA bioconversion is mediated by bacteria not necessarily associated with the breakdown of macromolecules such as carbohydrates during acidogenesis, but associated with the consumption of the produced OA. However, the spatial profiles of TBBPA and carbohydrates concentrations in the MR corroborate the hypothesis that TBBPA is indeed biodegraded during acidogenesis alongside carbohydrates consumption. The spatial concentration of TBBPA and carbohydrates in the MR show that their conversion reach 99.8 and 90.4%, respectively, within the first reactional hour (HRT of 6 h).

TBBPA biodegradation has been comprehended as essentially a cometabolic mechanism, in which microorganisms are able to transform it into products that do not yield energy for microbial growth (Harwood et al., 1998; Horvath et al., 1972) despite of studies in which TBBPA was successfully used as the sole carbon source when it was provided in high concentrations (in the order of mg L^{-1}) (An et al., 2011; McAvoy et al., 2016; Peng et al., 2017; Jiang et al., 2020). TBBPA reductive debromination occurring by cometabolic reactions can be justified by the fact that it is an ATP dependent process and that if only dehalogenation of the substrate is taking place and the aromatic ring remains intact, no carbon is provided for microbial growth (Häggblom et al., 1992; Fetzner, 1998). In addition, even if carbon and energy are provided from TBBPA biodegradation, it would not support biomass growth and maintenance at low concentrations (ng - µg L^{-1}). However, besides the reductive debromination via cometabolism, reductive dehalogenation as a respiratory process (dehalorespiration) may take place during the degradation of halogenated aromatic compounds, in a process in which TBBPA would be used as electron acceptor in an exergonic dehalogenation reaction, yielding energy that could be used for bacterial benefit (Fetzner, 1998; El Fantroussi et al., 1998; Zhang et al., 2005). In fact, metabolic reductive dehalogenation rates in general are several orders of magnitude higher than cometabolic rates (Fetzner, 1998). Anaerobic bacterial species may utilize several electron donors and acceptors in a respiratory system that involve hydrogenases, reductive dehalogenases, and electron carriers mediating electron transfer between them (Fetzner, 1998), with relaxed substrate specificity, meaning that a bacterial group may degrade a variety of halogenated compounds using different electron donors.

Many different exogenous carbon sources (yeast extract, carbohydrates, alcohols, and short chain fatty acids) have been indicated as suitable to support TBBPA degradation under anaerobic conditions (Iasur-Kruh et al., 2010; Peng and Jia, 2013; Peng et al., 2017; Wei et al., 2018; Lefevre et al., 2019; Jiang et al., 2020). This may be justified by the fact that the fermentation of organic compounds such carbohydrates and some fatty acids allows both the cometabolic and metabolic degradation of TBBPA by 1) the production of enzymes and cofactors lacking specificity and that are produced to degrade the exogenous organic macrocomponents, and 2) by providing the energy needed for the metabolic process of TBBPA reductive dehalorespiration from, for example, H_2 as an electron donor (Fetzner, 1998; Zhang et al., 2005). The simultaneous occurrence of both mechanisms may explain why so many different amendments support and/or enhance TBBPA biodegradation under anaerobic conditions as both the cometabolic (with no benefit for bacterial growth) and the metabolic (by providing energy) processes may occur simultaneously in complex microbial communities even at environmentally relevant concentrations. As both enzyme pools and H_2 are predominantly produced during acidogenesis, TBBPA anaerobic biodegradation occurs in this stage by combined mechanisms, different microbial groups, and throughout different routes.

In this study, carbohydrates consumption via the acetic acid metabolic route, which yields H_2 production, was positively correlated with TBBPA biodegradation. Iasur-Kruh et al. (2010) (Fetzner, 1998) firstly stated that TBBPA biodegradation was enhanced by carbon sources which yield hydrogen when fermented and that TBBPA degradation is most likely performed by syntrophic complex microbial communities. After their pioneer insights on the metabolic mechanism of TBBPA biodegradation under anaerobic conditions, other studies led to similar conclusion. For instance, lactic acid may accelerate the debromination process by its utilization as a carbon source for microbial growth and the generation of H_2 during its anaerobic bioconversion, acting as electron donor for TBBPA debromination and further BPA degradation (Wei et al., 2018; Lin et al., 2020). Furthermore, the addition of acetate as a cometabolic substrate delayed the degradation of TBBPA or was not the most suitable substrate for the microbial community (Lefevre et al., 2016).

Therefore, combining the findings of this study with the discussions reported in the literature (Fetzner, 1998; Lefevre et al., 2016; Wei et al., 2018; Lin et al., 2020). TBBPA biodegradation by anaerobic complex microbial communities at environmentally relevant concentrations occurs during acidogenesis via the cometabolism supported by non-specific

enzymes and cofactors. Additionally, electrons donors such as H_2 which are produced during the macrocomponents breakdown may support TBBPA-degrading metabolic reactions. In other words, carbohydrates consumption enhances TBBPA biodegradation by directly (cometabolism) and indirectly (dehalorespiration/metabolism) enabling both mechanisms.

Controversially, some studies (Peng and Jia, 2013; Lefevre et al., 2019) have discussed that TBBPA biodegradation was enhanced when using formic and acetic acid as the main electron donor and carbon source, respectively, which are direct substrates for methanogenesis. However, even though their results suggest that the degradation of TBBPA took place in the methanogenic stage and was favored by acetate and formate-consuming microbial groups, the carboxylic formic and acetic acids may favor TBBPA reductive debromination only by providing carbon to microbial growth which degrades TBBPA via metabolism, considering that the micropollutant was provided in concentrations in the order of mg L^{-1}.

In summary, the results from both AR and MR reactors indicate that TBBPA biodegradation is favored by carbohydrates breakdown by bacteria producing non-specific enzymes and by bacteria performing dehalorespiration, which is possibly supported due to H_2 production in exergonic reactions.

TBBPA: Removal or Biodegradation?

In order to differentiate biodegradation from removal, the adsorbed TBBPA was quantified in the biomass withdrawn from three different sections of the reactors' bed at the end of the operational period. Its concentration was 27.1 ± 7.7 µgTBBPA $gVSS^{-1}$ and 4.3 ± 0.4 µgTBBPA $gVSS^{-1}$ in the AR and MR, respectively. Detailed information on the adhered and suspended biomass and TBBPA concentration adsorbed along the bioreactors bed length is presented in Table B.1 (Appendix B). The prediction of the sorption capacity of TBBPA was not estimated by the solid–water distribution coefficients (k_d) (Ternes et al., 2004) because, when undergoing biodegradation, the dissolved and bound TBBPA fractions in a biological system are not necessarily at equilibrium (Potvin et al., 2012). The similar TBBPA concentrations quantified in the sludge in each of the reactors' sections indicate TBBPA saturation and the establishment of pseudo-steady-state conditions, as most of the TBBPA sorption was expected to take place at the bottom of the reactors if the sorption saturation was not yet reached (section 1 in Figure B.1).

The percentage of TBBPA degraded in the reactors was obtained by considering the influent and effluent TBBPA concentrations throughout the operational period, the TBBPA adsorbed in the suspended and adhered biomass, and the TBBPA possibly adsorbed in the effluent volatile suspended solids (VSS). According to the TBBPA mass balance, 86.8 ± 0.05 % and 97 ± 0.01 % of the removed TBBPA was in fact biodegraded and less than 3% and 1% was adsorbed in the AR and MR, respectively. These results suggest that even though TBBPA sorption onto the biomass occurred, biodegradation was the predominant phenomenon occurring in the bioreactors operated under environmentally relevant concentrations. The applied, removed, and biotransformed TBBPA were 0.87 ± 0.10, 0.78 ± 0.35, and 0.68 ± 0.11, and 0.36 ± 0.04, 0.35 ± 0.04, and 0.34 ± 0.04 gTBBPA m^{-3} d^{-1} in the AR and MR. The box plot graphs of TBBPA loading rates in presented in Figure B.2 (Appendix B) graphically expressing the adsorption factor on TBBPA removal from the acidogenic and methanogenic systems. Sorption was more expressive in the acidogenic reactor, most likely due to the lower bulk pH (5.5 ± 0.2) compared to the methanogenic reactor (6.9 ± 0.1), which favors TBBPA speciation to its undissociated form and plays an important role in the TBBPA partitioning to adsorptive surfaces (Potvin et al., 2012; Zhang et al., 2013). Moreover, the AR was operated under a greater organic and TBBPA loading rates compared to the MR to maintain acidogenic conditions (theoretical TBBPA loading rates of 1 and 0.4 g m^{-3} d^{-1} for AR and MR, respectively).

Knowledge on the adsorbed fraction of TBBPA is also important because even though the dissolved fraction of OMPs is often considered as the sole available and biodegradable fraction (Pomiès et al, 2013), biodegradation may occur in both phases (Xue et al., 2010; Gonzalez-Gil et al., 2018). The study conducted by McAvoy et al. (2016) supported this hypothesis for TBBPA, specifically. The authors investigated the biodegradation of TBBPA in digested sludge, soil, and sediments from freshwater environment with radiolabeled ^{14}C-TBBPA over 120 days. Despite the fact that most of the ^{14}C-TBBPA was immediately adsorbed onto the sludge solids, the amount of ^{14}C-TBBPA in the solid extracts decreased from 95% on day 0 to 13% by day 56 and 2% by day 120. These extracts also contained three unknown biotransformation products that were ultimately transformed into BPA, confirming TBBPA biodegradation after being sorbed onto solid particles.

In the present study, besides the smaller TBBPA loading rate applied in the MR and the higher pH value compared to the AR, the establishment of syntrophic microbial communities in the MR with hydrolytic, acidogenic, acetogenic, and methanogenic groups might have enhanced

TBBPA biodegradation and, consequently, a greater residual TBBPA concentration in the solid fraction was determined in the AR.

Degradation kinetics

Figure 3.3 shows the kinetic profiles of TBBPA and carbohydrates removal in both AR and MR and the parameters of the adjusted model are shown in Table 3.2. Even though the AR accumulated more biomass, the apparent specific first-order kinetic constant for TBBPA removal (k_{app}^{*} - considering the biomass in each reactor) of the MR was much greater. However, the reactors were operated under different HRT and organic and TBBPA loading rates to establish acidogenic and methanogenic conditions. Thus, the kinetic constants are not directly comparable.

Table 3.2 – First-order kinetic expressions estimated for the TBBPA and carbohydrates removal in the acidogenic (AR) and methanogenic (MR) reactors. C_0 and Cr in mg L^{-1} for carbohydrates and µg L^{-1} for TBBPA.

		C_o (µg L^{-1}, mg L^{-1})	C_r (µg L^{-1}, mg L^{-1})	k_{app} (h^{-1})	k_{app*} (h^{-1} $gVSS^{-1}$)	R^2
AR	TBBPA	52.75 ± 1.32	6.76 ± 0.51	8.64 ± 0.62	0.52	0.998
	Carbohydrates	325.47 ± 6.55	102.33± 1.45	18.97± 1.14	1.29	0.999
MR	TBBPA	156.53 ± 53.35	1.01 ± 0.06	8.78 ± 3.8	1.15	0.999
	Carbohydrates	323.72 ± 19.79	4.47 ± 4.07	2.92 ± 0.55	0.43	0.994

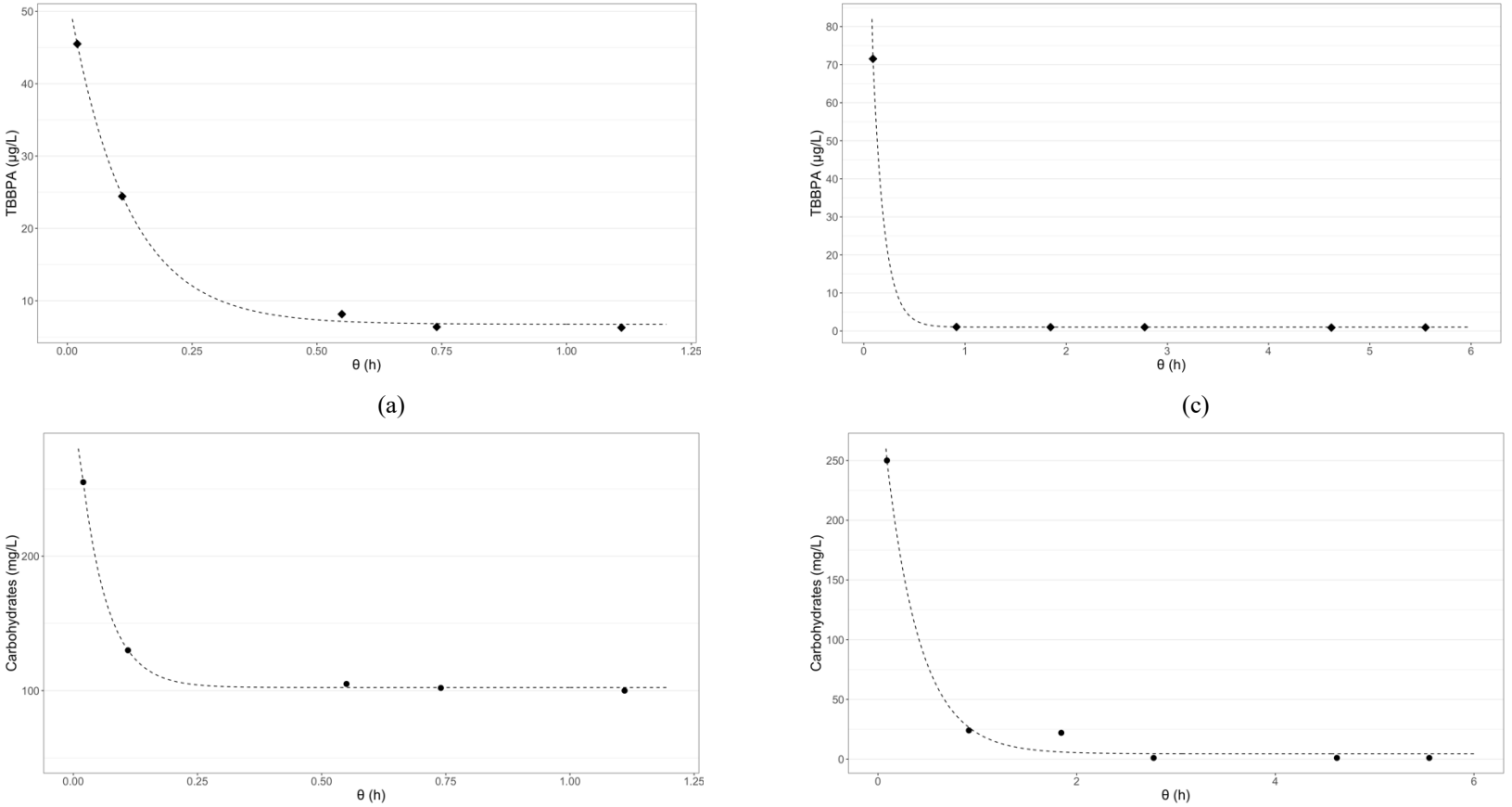


Figure 3.3 – Kinetic profiles of TBBPA and carbohydrates removal in the acidogenic (AR: a, b) and methanogenic (MR: c,d) reactors.

Biotransformation products and suggested metabolic pathways

TBBPA degradation in anaerobic biosystems is often associated with BPA accumulation, suggesting that no further degradation occurs beyond reductive debromination in this environmental condition (Ronen and Abeliovich, 2000; Voordeckers et al., 2002; Arbeli and Ronen, 2003; Arbeli et al., 2006; Iasur-Kruh et al., 2010; Lefevre et al., 2016; Lefevre et al., 2019). However, phenol was detected in the effluent of the bioreactors after the 47th operational day in concentrations of 20 ± 6.6 and 10.1 ± 11 μg L^{-1} in the AR and in the MR, respectively. Approximately 35 μg L^{-1} of total phenols could be produced from the influent TBBPA. However, as previously mentioned, TBBPA may be biodegraded after being sorbed and accumulated in the biomass (McAvoy et al., 2016), which may lead to effluent concentrations of degradation products greater than the stoichiometrically estimated. The possible metabolic pathways by which TBBPA was further biodegraded are presented in Figure 3.4.

Among processes observed under anaerobic conditions, the main TBBPA degradation routes are based on dehalogenation (reductive and oxidative), beta-scission, and ring cleavage reactions (Table 3.3).

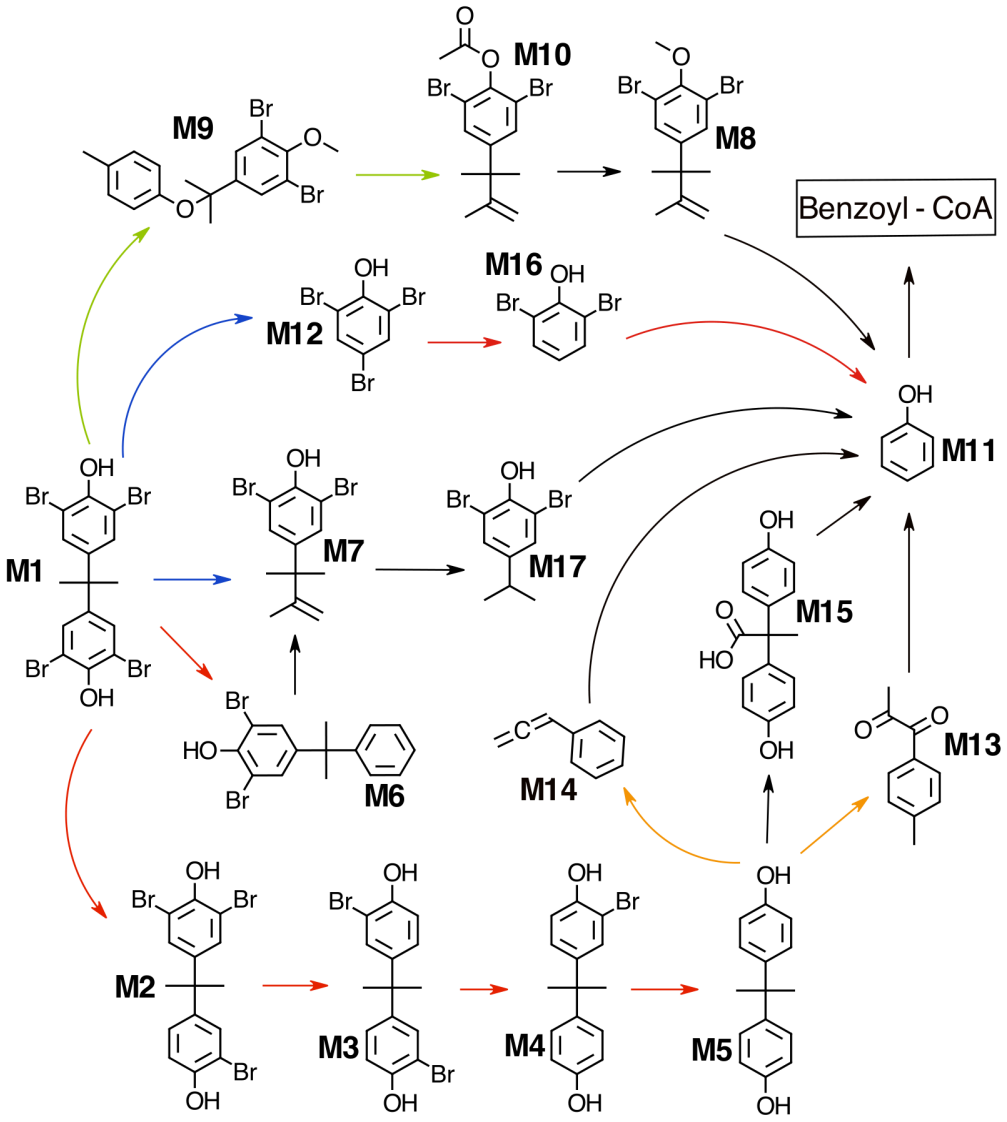


Figure 3.4 – Possible metabolic pathways of TBBPA biodegradation to phenol and the formation of benzoyl-CoA. Biochemical reactions: oxidative decomposition (green), beta-scission (blue), debromination (red), carbon-carbon bond or ring cleavage (orange). The arrows in black represent multiple or not mentioned reactions such as decomposition, dehydrogenation, hydrogenation, deprotonation, and substitutions.

Table 3.3 – Possible metabolic pathways and intermediates products leading to phenol generation from TBBPA biodegradation.

Concentration	Condition	Exogenous carbon source	Pathways	Main transformation products or Intermediates	Reference
50 μg L^{-1}	Anaerobic	Domestic sewage**	-	Phenol (**M11**)	This work
25 - 500 μg L^{-1}		Glucose	Oxidation, debromination, and dehydrogenation.	2,6-dibromo-4-(1-methyl-1-phenylethyl) phenol (**M6**), 2,6-dibromo-4-(prop-1-en-2-yl) phenol (**M7**), 1,3-dibromo-2-met\hoxy-5-(prop-1-en-2-yl) benzene (**M8**)	Fan et al. (2017)
		Glucose and HA		1,3-dibromo-2-methoxy-5-(2-(p-tolyloxy) propan-2-yl) benzene (**M9**) 2,6-dibromo-4-(prop-1-en-2-yl) phenyl acetate (**M10**)	Chen et al. (2019)
0.5 mg L^{-1}		Formate	Oxidation, debromination, and Beta-scission.	2,6-dibromo-4-(1-methyl-1-phenylethyl) phenol (**M6**), 2,6-dibromo-4-isopropyl-phenol (**M17**) 2,6-dibromophenol (**M16**) and phenol (**M11**)	Fan et al. (2020)
		Glucose	Oxidation, debromination, TBBPA-cleavage	less brominated compounds, TBP (**M12**), and oxaloacetic acid	Peng et al. (2017)
10 mg L^{-1}		Lactate	Oxidative hydrolysis, debromination, and Beta-scission	BPA (**M5**) further degraded to 4-(allene) phenol (**M14**) and 2,2-bis(4-hydroxyphenyl) propanoic acid (**M15**)	54
2.5 mg L^{-1}		Acetate			Lin et al. (2020)*
4 mg L^{-1}					Lefevre et al. (2016)*
43.5 mg $L^{-1}L$		Ethanol			Lefevre et al. (2019)
50 mg L^{-1}		Glucose			Iasur-Kruh et al. (2010), Arbeli et al. (2006)
100 mg L^{-1}		Peptone-glucose yeast extract	Reductive debromination	BPA (**M5**)	Arbeli and Ronen (2003)*
122.4 mg L^{-1}					Ronen and Abeliovich (2000)*
50 μg kg^{-1} (dw)		None			Voordeckers et al. (2002)
10.78 mg kg^{-1} (dw)					McAvoy et al. (2016)
20 mg L^{-1}		Lactate			Jiang et al. (2020)*
0.5 mg L^{-1}		None	Reductive debromination and benzoyl-CoA reaction	BPA (**M5**), 3, 4-dihydroxymandelic acid, beta resorcylic acid, acetylbenzoyl (**M13**), acetophenone, and oxaloacetic acid	Wei et al. (2018)
					Peng et al. (2013)

*TriBBPA (**M2**), DiBBPA (**M3**), and MonoBBPA (**M4**) were also detected; **Lab-made domestic sewage.

The prevailing pathway for TBBPA biodegradation is that the halogen substituents are removed one by one through either hydroxylation or reductive debromination, and then the aromatic ring is cleaved (Heider and Fuchs, 1997). Yet, the TBBPA degradation pathways proposed by many authors (An et al., 2011; Gu et al., 2016; Fan et al., 2017; Gu et al., 2018; Gu et al., 2019; Liang et al., 2019; Fan et al., 2020) suggest that the bromide atoms may be removed after carbon-carbon bond cleavage.

Aromatic ring cleavage is a critical step on the degradation of halogenated aromatic compounds and intermediates, such as catechols, which are readily attacked oxidatively in aerobic degradation. Under anaerobic environments, in the cleavage of aromatic rings, the carbon-carbon bonds are broken by reduction and the ring is subsequently opened hydrolytically. The most common central intermediate in anaerobic aromatic metabolism is benzoyl-CoA, for which the common key enzyme for ring reduction in these metabolic routes is benzoyl-CoA reductase (dearomatising) (Heider and Fuchs, 1997; Harwood et al., 1998).

Even though it is not possible to suggest which TBBPA metabolic pathway prevailed in this study, TBBPA biodegradation went further than reductive debromination, which is likely due to the complex microbial consortia involved in the process. Many bacterial genera have been associated to TBBPA degradation activity and there is a predominance of the Gram-negative phyla *Bacterioidetes, Spirochaetes*, and *Proteobacteria* (Zhang et al., 2013 ; Lefevre et al., 2016 ; Yang et al. 2016 ; Fan et al., 2017 ; Peng et al. 2017 ; Fan et al., 2020; Jiang et al., 2020) followed by the Gram-positive bacteria belonging to the phyla *Firmicutes* (*Bacilli* and *Clostridia*) and *Actinobacteria* (*Rhodococcus*) (An et al., 2011; Zhang et al., 2013; Lefevre et al. 2016 ; Yang et al. 2016; Fan et al., 2017; Fan et al., 2020 ; Lin et al., 2020).

Amplicon and shotgun metagenomic sequencing was performed to analyze the microbiome of the inoculum used in this study (Delforno et al., 2017). *Proteobacteria, Firmicutes,* and *Bacterioidetes* are the most abundant phyla in the inoculum, which corroborates with the literature on the possible bacterial groups responsible for TBBPA bioconversion.

Conclusion

Tebrabromobisphenol A anaerobic biodegradation occurs during acidogenesis. This phenomenon was observed in both acidogenic and methanogenic reactors, being positively and strongly correlated to carbohydrates removal and acetic acid production. The results of this study

suggest that besides the established TBBPA-degrading cometabolism, dehalorespiratory processes could be involved in TBBPA bioconversion. It is hypothesized that the cometabolism was favored by non-specific enzymes produced in order to degrade the organic macrocomponents, and the metabolic-respiratory process was favored by electrons donors such as H_2 produced during acidogenesis. Even though TBBPA sorption onto the biomass occurred in both systems, biodegradation was the main phenomenon driving the micropollutant removal from the wastewater. This is the first research to relate the anaerobic biodegradation of TBBPA at environmentally relevant concentrations by complex microbial communities to a specific stage of the anaerobic digestion process.

Author Contributions

W.V. Macêdo: conceptualization, methodology, data curation, formal analysis, investigation, writing of original draft, review, and editing; G. H. D. Oliveira: conceptualization, methodology, investigation, writing of original draft, review, and editing; M. Zaiat: conceptualization, methodology, resources, writing, review, editing, visualization, supervision, project administration, and funding acquisition.

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Chapter 4

Tetrabromobisphenol A (TBBPA) biodegradation in acidogenic systems: one step further on where and who

Abstract. The occurrence of brominated flame retardants such as Tetrabromobisphenol A (TBBPA) in water bodies poses a serious threat to aquatic ecosystems. Degradation of TBBPA in wastewater has successfully been demonstrated to occur through anaerobic digestion (AD), although the involved microorganisms and the conditions favouring the conversion remains unclear. In this study, it was observed that bioconversion of TBBPA did not occur during the hydrolytic stage of the AD, but during the strictly fermentative stage. Bioconversion occurred in hydrolytic-acidogenic as well as in strictly acidogenic continuous bioreactors. This indicates that the microorganisms that degrade TBBPA benefit from the electron flux taking place during glycolysis and further transformations into short-chain fatty acids. The degradation kinetics of TBBPA was inversely proportional to the complexity of the wastewater as the apparent kinetics constants were 2.11, 1.86, and 0.52 $h^{-1} \cdot gVSS^{-1}$ for glucose, starch, and domestic sewage as carbon source, respectively. Additionally, the micropollutant loading rate relative to the overall organic loading rate is of major importance during the investigation of cometabolic transformations. The long-term exposure to TBBPA at environmentally realistic concentrations did not cause any major changes in the microbiome composition. Multivariate statistical analysis of the evolvement of the microbiome throughout the incubation suggested that *Enterobacter* spp. and *Clostridium* spp. are the key players in TBBPA degradation. Finally, a batch enrichment was conducted, which showed that concentrations of 0.5 $mg \cdot L^{-1}$ or higher are detrimental to *Clostridium* spp., even though these organisms are putative TBBPA degraders. The *Clostridium* genus was outcompeted by the *Enterobacter* and *Klebsiella* genera, hereby highlighting the effect of unrealistic concentrations frequently used in culture-dependent studies on the microbial community composition.

Keywords: Tetrabromobisphenol A; Hydrolysis; Enterobacter; Clostridium; Cometabolic degradation.

Introduction

Most aspects of our daily life involve products such as plastics, electronic devices, epoxy resins, and building materials. In those products, flame retardants are often applied to meet fire safety requirements. Among the different classes of plastic additives, Tetrabromobisphenol A (TBBPA) is one of the most widely used brominated flame retardants. TBBPA is considered a hazardous organic micropollutant (OMP) and has been detected in natural water bodies in concentrations up to 5 $\mu g \cdot L^{-1}$ (Yang et al., 2012; Xiong et al., 2015; Xu et al., 2021) and up to 1329 $ng \cdot g^{-1}$dw in sewage sludge samples (dry weight, dw) (Gorga et al., 2013). As most of the halogenated flame retardants, TBBPA can enter wastewater treatment plants via domestic sewage contaminated with consumer products, manufacturing facilities wastewater, landfill leachate, and precipitation (Xu et al., 2021).

TBBPA has been classified as an endocrine disruptor as it causes genetic mutation in frogs (Veldhoen et al., 2006), maternal transmission between fish generations (Nyholm et al., 2008), and affects mussels' physiology by chronic exposure to environmentally relevant concentrations (Wang et al., 2021). Additionally, TBBPA causes immunotoxic and neutorotoxic effects on aquatic organisms (Covaci et al., 2009). As a consequence, this OMP has been listed as a restricted substance by the European Union's Restriction of Hazardous Substances Directive (Xu et al., 2021).

TBBPA biodegradation has been investigated over the past 20 years (Ronen and Abeliovich, 2000; Iasur-Kruh et al., 2010; An et al., 2011; Zu et al., 2014; Lefevre et al., 2016; 2019; Li et al., 2016; Gu et al., 2016; 2018; 2019; Lin et al., 2020), but nearly all bioconversion studies have been conducted at artificially high concentrations ($mg \cdot L^{-1}$). Furthermore, most of these studies have used pure or enriched cultures in laboratory settings, and to the best of our knowledge, none so far with culture-independent approaches. High TBBPA concentrations may induce metabolic pathways and gene expressions that would not otherwise occur or lead to inhibition, highlighting the relevance of investigating the pollutant degradation at more realistic concentrations. Additionally, the information on what promotes this micropollutant degradation relies mostly on process efficiencies correlating removal rate and half-life with response to the variations on pH, carbon sources, temperature, bioaugmentation, and other pertubations. However, the microbial community responsible for TBBPA bioconversion in wastewater treatment plants remains little explored.

The anaerobic digestion (AD) has been extensively studied in relation to the treatment of wastewater, and it is an efficient and feasible technology for the conversion of macro and micropollutants (Castellano-Hinojosa et al., 2018). Hydrolysis is a crucial step in the AD process, in which several enzymatically driven reactions break down complex macromolecules into monomers. As cometabolic conversion of OMPs involves non-specific enzymes (Fetzner, 1998; Carneiro et al., 2020), complex carbon sources in mixed cultures may favour the bioconversion of OMPs, while readily fermentable carbon sources primarily hydrolyse into glucose or similar carbohydrates.

The metabolic routes during hydrolysis and acidogenesis depend on the substrate composition, and for that reason, cometabolic studies comparing simple and complex carbohydrate sources may shed light on the role of acidogenic microorganisms in TBBPA biodegradation. Recently, it has been evidenced that TBBPA biodegradation at environmentally relevant settings occurs during acidogenesis, being positively and strongly correlated to the removal of carbohydrates and acetic acid production (Macêdo et al., 2021). Even though the micropollutant bioconversion was related to acidogenesis, the role of the hydrolytic stage in TBBPA bioconversion and the microorganisms potentially associated with this transformation in mixed communities remain unclear.

The identification of the taxonomic groups associated to OMP biodegradation may be achieved by temporal correlations of the microbiome composition and the pollutant's degradation in continuous systems (Liang et al., 2021). The abundance variation in a bacterial community throughout time exposure to TBBPA at low concentrations along with its bioconversion efficiency may elucidate bacterial groups and biodegradation patterns that would occur in environmental settings. Knowledge of TBBPA degraders may also lead to a better comprehension of the environmental fate of other flame retardants and allow further optimization of wastewater treatment management.

Therefore, we hypothesized that the hydrolytic activity in AD systems would benefit TBBPA bioconversion and that the possible TBPPA degraders could be identified by multivariate statistical analysis by combining temporal biomass sampling and sequencing with the performance of the bioreactors. This could be achieved by evaluating TBBPA degradation during starch breakdown and further fermentation (hydrolysis + acidogenesis), compared to a strictly glucose-fermentative system (acidogenesis). For this purpose, a strictly acidogenic (RAc) and a hydrolytic-

acidogenic (RHAc) anaerobically structured bed reactor (ASTBR) were operated at the same organic and TBBPA load rates. To uncover the bacterial populations potentially involved in the pollutant breakdown, adhered and suspended biomass were withdrawn weekly and analyzed by amplicon sequencing of the 16S rRNA gene.

Material And Methods

Bioreactor's description and operational conditions

The bench-scale reactors used in this study were two anaerobically structured bed reactors (ASTBR) previously used by Macêdo et al. (2021). Both of them were identical, made of acrylic material, length 65 cm, and an internal diameter of 8 cm. The reactor's bed contained 6 vertical strips of polyurethane foam (length of 52 cm and base of 1 cm^2). The characterization of the support material was as follows: specific surface area of 43.8 $m^2 \cdot g^{-1}$, porosity of 92%, and apparent density of 23 $g \cdot L^{-1}$ (Silva et al., 2006). A schematic description of the bioreactors is presented in Figure B.1 (Appendix B).

The strictly acidogenic reactor (RAc) was fed a readily fermentable carbon source (glucose, 937.5 $mg \cdot L^{-1}$) and the hydrolytic-acidogenic reactor (RHAc) was fed a more complex carbon source (starch, 890.0 $mg \cdot L^{-1}$). Both reactors were operated with the same theoretical chemical oxygen demand (COD) of 1000 mg $O_2 \cdot L^{-1}$ for comparison purposes. TBBPA was added to a final concentration of 50 $\mu g \cdot L^{-1}$ in the feed solution (see Appendix C for detailed description), which was prepared every 48 hours and kept refrigerated (+ 4 ºC).

The reactors were inoculated with anaerobic sludge from a full-scale up-flow anaerobic sludge blanket (UASB) reactor located at São Paulo, Brazil. In order to inhibit methanogenic activity, the inoculum was pre-treated by the acid shock method also described elsewhere (Penteado et al., 2013; Macêdo et al., 2021). Both reactors were operated for 85 days with hydraulic retention time (HRT) of 2.4 h, volumetric organic load rates (OLR) of 12 kg $COD \cdot m^{-3} \cdot d^{-1}$, and controlled temperature of 30 ºC. The stock solution of TBBPA was prepared in methanol and stored in a freezer (−20 ºC).

Enrichment

A 5-g biomass sample was collected from the RAc in the final operational week and added to 250 mL mineral medium (MM) containing 0.5 $mg \cdot L^{-1}$ of TBBPA and 1 $g \cdot L^{-1}$ of glucose as the carbon source at a concentration of 1 $g \cdot L^{-1}$. After 5 days of incubation at 30 °C with shaking at 150 rpm, 10 % (v/v) of the culture was inoculated into 250 mL MM with increasing TBBPA levels. Each cycle lasted 5 days as it was the period in which glucose was completely consumed. The TBBPA concentrations increased from 1.5 to 5 $mg \cdot L^{-1}$ to enrich the TBBPA degrading bacteria. The final concentration of TBBPA was measured at the end of each cycle, as well as the organic acids (OA) and biogas composition. At the end of each cycle, the content was centrifuged at 5366 *xg* for 15 minutes. The pellets were rinsed with PBS solution three times and stored at –20 ºC until further DNA extraction.

Biomass sampling

Both adhered and suspended biomass were withdrawn weekly from the same section (section 01) (Figure B.1) (Appendix B) of the reactors. For sampling the suspended biomass, 50 mL of the reactors' bulk were centrifuged at 5366 *xg* for 15 minutes. For the adhered biomass, approximately 1 cm^3 of the support material was withdrawn from the reactor bed. The biomass was detached from the polyurethane foam by vigorous shaking in a closed container with MilliQ water and glass beads. The process was repeated until the foam was clear, and the content was centrifuged at 5366 *xg* for 15 minutes. Both pellets (adhered and suspended biomass) were rinsed with phosphate-buffered saline (PBS) solution three times and stored at –20 ºC.

Chemicals and analytical methods

Tetrabromobisphenol A (TBBPA, 4,40-Isopropylidenebis (2,6-dibromophenol), 97% purity, CAS 79-94-7) (Sigma-Aldrich) and all other chemicals used in the feed and nutrients' solution were at least of analytical grade. Labelled TBBPA ($^{13}C_{12}$ TBBPA), which was used as the internal standard in the analytical method for TBBPA quantification, was purchased from Wellington Laboratories (Guelph, Canada).

TBBPA quantification was performed by dispersive liquid-liquid microextraction (DLLME), followed by liquid chromatography-tandem mass spectrometry (LC–ESI–MS/MS) analysis. An Agilent Technologies 1260 Infinity chromatograph equipped with an Agilent

Technologies Poroshell 120 EC-C18 column (50 mm × 3.0 mm; 2.7 μm) was used for the chromatographic separation. Tandem mass spectrometric analysis was performed using a 5500 QTRAP hybrid mass spectrometer (ABSciex) equipped with a TurboV^{TM} ion source, operated in the negative electrospray mode (ESI-). Details on the sample preparation, extraction procedure, analytical conditions, and ESI-MS parameters are described elsewhere (Macêdo et al., 2020).

Physico-chemical analyses and TBBPA removal were performed twice a week. The suspended solids concentration (2540-E), chemical oxygen demand (COD) (5220-D), and pH (4500-H+ B) were measured in accordance with the Standard Methods (APHA, 2017), and carbohydrates concentration was determined according to DuBois et al. (1956). Samples were collected, filtered in Combi syringe filters with a coarse glass fiber prefilter and a small-pore membrane as the main filter (polytetrafluoroethylene) (1.0/0.20 μm), and analyzed on the same day. The organic acids (lactic, formic, acetic, propionic, butyric, isobutyric, valeric, and isovaleric) were quantified by a high-performance liquid chromatograph (HPLC, Shimadzu®) equipped with a UV-diode array detector (SDP-M10 AVP), refraction index detector (RID-10A), and an Aminex HPX-87H column (300 mm, 7.8 mm, BioRad®) (Penteado et al., 2013). A gas chromatograph (GC-2010, Shimadzu®) equipped with a thermal conductivity detector with argon as the carrier gas and a CARBOXEN® 1010 Plot (30 m, 0.53 mm) column was used for analyzing the biogas composition as described elsewhere (Penteado et al., 2013).

DNA extraction and 16S rRNA gene amplicon sequencing

The samples from the reactor with best performance on TBBPA bioconversion as well as biomass from the other reactor (day 85) were analyzed by amplicon sequencing. DNA was extracted from approximately 500 mg (wet weight) of biomass using the FastDNA® SPIN®Kit for soil (MP Biomedicals). DNA concentrations were measured using the Quant-IT BR DNA Assay (Thermo Fisher Scientific) on an Infinite M200 PRO (TECAN, Switzerland) plate reader. The V4 variable region of the 16S rRNA was amplified using primer set 515F/806R (515F: 5'-GTGCCAGCMGCCGCGGTAA-3' and 806R: 5'-GGACTACHVGGGTWTCTAAT-3') (Caporaso *et al.*, 2011) and sequenced using the Illumina platform. Equimolar concentrations were sequenced on a MiSeq (Illumina) using MiSeq reagent kit v3 (2 x 300 PE). All amplicon data is available at the European Nucleotide Archive (ENA) under project accession number PRJEB49191.

Data analysis

The spatial profiles in the reactors were obtained by collecting samples along the reactors' length in the final operational week, representative of the achieved pseudo-steady state. The reactors' flow pattern is plug flow (Carneiro et al., 2019), and for that reason, samples collected in the points along the reactor length (Figure S1) represented different HRTs, from 0 to 2.4 h. In these samples, TBBPA and carbohydrates concentrations were quantified. A first-order kinetic model with a residual concentration (Camargo et al., 2002) was adjusted to the experimental data (Equation 1, Chapter 3).

The statistical analyses (ANOVA, Levene's test, t-test, Pearson's correlation matrix, and first-order kinetic model fitting) were performed using SPSS® and RStudio (version 1.2.5001, http://www.rstudio.com). Analyses of the amplicon sequenced data were performed via Rstudio using the ampvis2 package (Andersen et al., 2018). Alpha diversity was measured using the observed amplicon sequence variant (ASV) and Shannon-Weaver index (Shannon, 1948) using the phyloseq package (McMurdie and Holmes, 2013). Beta diversity was investigated using principal coordinates analysis (PCoA) with Bray-Curtis dissimilarity. Correlations between the main identified genera and operational parameters were generated using Spearman's correlations.

Results And Discussion

Hydrolysis is a negligible step in acidogenic systems for TBBPA bioconversion

Both ASTBR reactors were continuously operated for 85 days with an HRT of 2.4 h. The averages of overall removal of TBBPA, organic matter (carbohydrates and chemical oxygen demand, COD), and the main OA produced throughout the operational period are presented in Table 4.1. All TBBPA removed was considered to be bioconverted as its sorption onto the biomass has previously been found to be negligible (Macêdo et al., 2021). All reactor performance analyses were processed from day 15 onwards, after the start-up period was considered to be over and the reactors were in steady-state conditions.

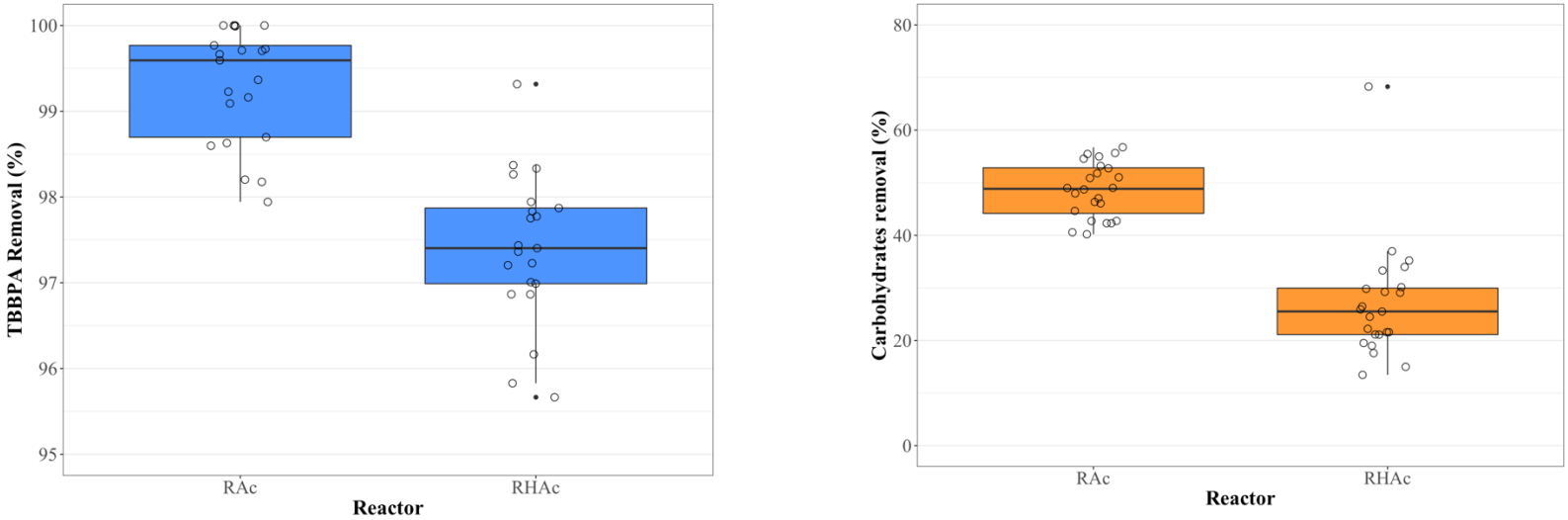
Table 4.1 – Removal of TBBPA, organic matter, and production of organic acids in the strictly-acidogenic (RAc) and hydrolytic-acidogenic (RHAc) reactors.

Parameters		RAc	RHAc
pH	Influent	7.2 ± 0.1	7.3 ± 0.1
	Effluent	4.7 ± 0.1	5.3 ± 0.7
TBBPA	Influent (μg·L^{-1})	45.2± 9.6	49.2 ± 3.9
	Effluent (μg·L^{-1})	0.3 ± 0.2	1.5 ± 0.6
	Removal (%)	99.3 ± 0.6	97.2 ± 1.1
Organic Matter	Carbohydrates removal (%)	50.3 ± 6.7	30.2 ± 13.1
	COD removal (%)	9.1 ± 3.9	5.5 ± 2.7
Organic acids production	HAce (mg·L^{-1})	62.3 ± 29.6	59.6 ± 37.1
	FA (mg·L^{-1})	9.2 ± 4.6	7.2 ± 3.8
	HBut (mg·L^{-1})	29.8 ± 34.2	31.9 ± 37.6
	HLac (mg·L^{-1})	53.9 ± 19.9	9.1 ± 8.4

HAce: Acetic acid; FA: Formic acid; HBut: Butyric acid; HLac: Lactic acid.

The acidification performance of an anaerobic system is calculated by the ratio between the COD of the OA produced and the influent COD ($COD_{OA}/COD_{influent}$, %) (Carneiro et al., 2020; Menzel et al., 2020). The RAc and RHAc acidification performances were 17.9 and 13.4 %, respectively, and the main OA produced were identified to be mainly acetic, butyric, lactic, and formic acids. Lactic acid production was favoured in the RAc probably due to higher concentrations of readily bioavailable glucose, leading to a lower pH in the bulk. With the exception of lactic acid, there is no statistical difference between RHAc and RAc in acetic, formic, and butyric acid production as determined by Levene's test for equality of variances and t-test for equality of means (Table C.1 and C.2) (Appendix C). Aside from lactic acid production, the fermentation routes were not affected by the different carbon sources. The biogas of both reactors was analyzed once a week and was composed of H_2 and CO_2 throughout the steady-state period, and CH_4 was not detected after the start-up period.

In order to evaluate the effect of the hydrolytic stage on TBBPA bioconversion, the correlations between TBBPA degradation, the acidification efficiency, and the microbial composition were investigated using multivariate and descriptive statistical approaches. The results of this research suggest that, by comparing the RAc and the RHAc, TBBPA biodegradation was not limited by the lack of the hydrolytic stage of the anaerobic digestion. On the contrary, in the system fed with readily bioavailable glucose, a higher TBBPA removal efficiency was obtained (Figure 4.1). TBBPA biodegradation in the RAc (99.3 ± 0.6 %, n = 28) was significantly ($p < 0.05$) higher, compared to the RHAc (97.2 ± 1.1 %, n = 28) as determined by Levene's test and t-test (Table C.3 and C.4) (Appendix C)).



(a) (b)

Figure 4.1 – TBBPA (a) and carbohydrates (b) removal (%) in the strictly-acidogenic (RAc) and hydrolitic-acidogenic reactors (RHAc). Open circles are the distribution of the samples throughout the operational period. Dots show outliers.

In the RHAc, glucose metabolization was limited by starch hydrolysis, which led to lower efficiency on carbohydrate removal, compared to the RAc, which were 30.2 ± 13.1 % and 50.3 ± 6.7 %, respectively (measured as total carbohydrates). However, the limited glucose metabolism still led to the formation of organic acids sufficiently to support TBBPA cometabolism. This can be attributed to the difference in orders of magnitude between the carbohydrate and TBBPA levels in the reactors. The complete debromination of TBBPA to BPA requires, according to the theoretical stoichiometry, 8 moles of electrons per mole of TBBPA reduced. Thus, an initial TBBPA concentration of 50 $\mu g \cdot L^{-1}$ would demand only around 5.9 $\mu gCOD \cdot L^{-1}$ for a complete reduction to BPA. In the RHAc reactor, an estimate of 55 mg $COD \cdot L^{-1}$ of the influent COD was not accounted for in the effluent stream (Table 4.1).

Despite the near-negligible COD removal, the amount of reducing power made available is several folds higher than that needed for complete TBBPA reduction. The micropollutant loading rate relative to the overall organic loading rate is important when investigating cometabolic transformations, and this is often overlooked. Therefore, the hydrolytic step in the AD system appeared only to affect the TBBPA conversion by limiting pyruvate formation and further pyruvate transformations into short-chain fatty acids, alcohols and biohydrogen. It can be hypothesized that the bioreactors' efficiency in converting TBBPA is inversely proportional to the complexity of the wastewater in AD systems by comparing the effect of limited acidification towards TBBPA biodegradation with previous results (RAc, glucose > RHAc, complex carbohydrates > AR, domestic sewage) (Macêdo et al., 2021). Hence, the hydrolytic activity in AD systems is a negligible step for TBBPA bioconversion at environmentally relevant concentrations.

Based on the fate of 21 OMPs during AD, it has previously been shown that the enzymatic activities involved in hydrolysis did not significantly contribute to the bioconversion of OMPs, while acidogenesis was the key step (Carneiro et al., 2020). For TBBPA specifically, products of acidic fermentation (ethanol and lactic acid) were suggested as the most suitable carbon sources to enhance the pollutant bioconversion (Iasur-Kruh et al., 2010; Wei et al., 2018; Lin et al., 2020), even though no specific connections with acidogenesis were stated. The acidogenesis of monosaccharides involves (1) pyruvate formation, (2) further pyruvate transformation into organic acids and alcohols, and (3) gaseous and non-gaseous products transformation (Angenent et al., 2004; Sikora et al., 2013; 2019). These steps are driven by various functional groups of organisms, which are the best candidates on TBBPA cometabolic conversion. However, the enzymatic

mechanism by which the cometabolism of TBBPA occurs is still unexplored and requires more in-depth studies.

Assuming the acetic acid route as the main degradation pathway of glucose in this study for both RAc and RHAc (Angenent et al., 2004), the reducing power produced during hexose metabolism (glycolysis) and pyruvate fermentation (acetate formation) may be used to reduce TBBPA (electron-acceptor molecule) into less harmful compounds. This can be assumed based on the fact that hydrogen production takes place involving enzyme-catalyzed transfer of electrons from an intracellular electron carrier molecule to protons. As protons are poor electron acceptors, TBBPA may be a competing electron acceptor molecule. In a previous study, Macêdo et al. (2021) has suggested that the TBBPA biodegradation in acidogenic systems occurs by combined mechanisms in complex microbial communities: with and without benefit for bacterial growth. It has been hypothesized that many amendments support TBBPA biodegradation by the utilization of carbon sources for microbial growth and the H_2 produced during fermentation and used as electron donor in reductive reactions (Wei et al., 2018; Lin et al., 2020; Macêdo et al., 2021).

Degradation kinetics

The parameters of the adjusted kinetic model for the spatial profiles of TBBPA and carbohydrate removal in both RAc and RHAc are presented in Table 4.2.

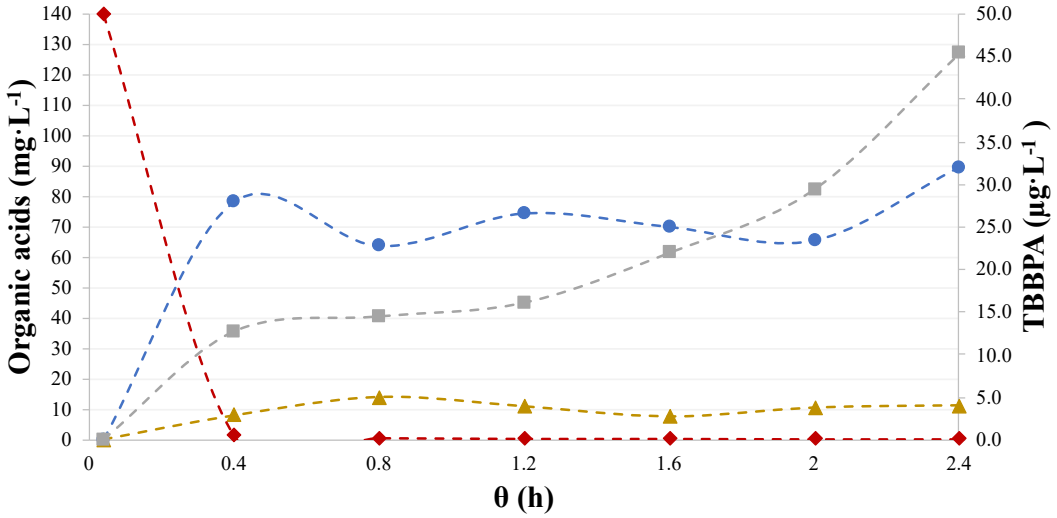
Table 4.2 – First-order kinetic expressions estimated for the TBBPA and carbohydrates removal in the strictly acidogenic reactor (RAc) and the hydrolytic-acidogenic reactor (RHAc). C_0 and Cr in $mg \cdot L^{-1}$ for carbohydrates and $\mu g \cdot L^{-1}$ for TBBPA.

		C_o ($\mu g \cdot L^{-1}$, $mg \cdot L^{-1}$)	C_r ($\mu g \cdot L^{-1}$, $mg \cdot L^{-1}$)	k^{app} ($h^{-1} \cdot g \cdot VSS^{-1}$)	R^2
RAc	TBBPA	50.62 ± 0.06	0.13 ± 0.02	2.11	0.999
	Carbohydrates	1003.17 ± 19.25	460.61 ± 10.08	0.57	0.995
RHAc	TBBPA	50.45 ± 0.35	1.25 ± 0.17	1.86	0.999
	Carbohydrates	994.78 ± 17.94	632.65 ± 24.76	0.28	0.955

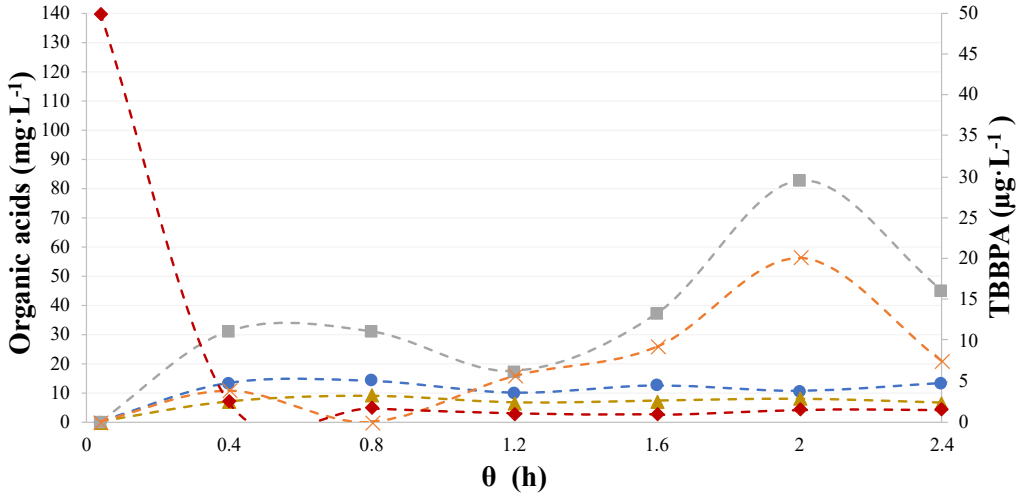
The apparent first-order kinetic constant for TBBPA removal (k^{app} - considering the biomass in each reactor on the final operational day in gVSS) was slightly higher in the RAc (2.11 $h^{-1} \cdot gVSS^{-1}$), compared to the RHAc (1.86 $h^{-1} \cdot gVSS^{-1}$), as well as the k^{app} for carbohydrate removal, 0.57 and 0.28 $h^{-1} \cdot gVSS^{-1}$, respectively. In an acidogenic system treating domestic sewage with the same organic and TBBPA loading rates, a k^{app} of 0.52 $h^{-1} \cdot gVSS^{-1}$ was reported for TBBPA removal

(Macêdo et al. 2021). The degradation kinetics in that system was slower, most likely due to the complexity of the wastewater, compared to readily bioavailable glucose and a lower carbohydrate loading rate.

The spatial profile of TBBPA removal and the organic acids in the RAc and in the RHAc are shown in Figure 4.2. In the first section of the reactors, the acetic and formic acid production had a similar profile in both systems. The bioavailability of glucose in the RAc led to a slightly faster degradation of TBBPA and high production of acetic acid, which was 35.7 and 30.9 $mg \cdot L^{-1}$ for the RAc and the RHAc, respectively. At that point, TBBPA had already reached residual concentrations of 0.48 and 2.44 $\mu g \cdot L^{-1}$ in the RAc and RHAc, respectively. Thus, the key groups of hydrolyzing bacteria involved in the degradation of organic matter had likely little influence on enhancing the kinetics of TBBPA bioconversion, which corroborates the results previously discussed.



(a)



(b)

Figure 4.2 – Profile of TBBPA and organic acids concentration in the strictly-acidogenic reactor (a) and in the hydrolytic-acidogenic reactor (b). (●) Lactic acid; (▲) Formic acid; (■) Acetic acid; (◆) TBBPA; (×) Butyric acid. The dashed lines are included as guide to the eyes and have no relation to fitted model.

Influence of TBBPA exposure on microbial community richness and diversity

Amplicon sequencing of the V4 variable region of the 16S rRNA gene revealed microbial communities with high complexity and diversity. The average reads per sample were 101,791 ± 47,153 reads, and a total of 7171 unique ASV were identified. The RAc had an average richness of 6.20 ± 0.43 and 5.99 ± 0.42 (Shannon Index) for adhered and suspended biomass, respectively. For the RHAc, richness index of the final sample was higher (6.86 for adhered biomass and 7.03 for suspended biomass), compared to the average Shannon Index of the RAc due to the starch supporting a more diverse bacterial community.

Major temporal changes in the RAc microbiome occurred in the first 20 days, most likely due to adaptation to acidogenic conditions, and more gradual shifts in the relative abundance of the main genera occurred until day 51, which might be related to the long-term exposure to TBBPA. A shift in the bacterial composition can be observed from week 6 to week 8 (day 44 to day 58), along with a decrease in the TBBPA removal in this period. During this period, the main disturbance was observed in the *Enterobacteriaceae* family, with a major increase in the *Salmonella* genus, and a decrease in relative abundance in the *Enterobacter* and *Klebsiella* genera.

During the final four weeks, the microbial community composition was relatively stable, even though a significant increase in the relative abundance of the genera *Salmonella* and

Sporolactobacillus was observed. At the end of the experiment, the dominant genera (>1 % of total reads) in the microbial community were the *Clostridium*, *Sporolactobacillus*, *Enterobacter*, *Klebsiella*, and *Salmonella.* The main genera (20 most abundant) present in the samples from RAc and in the final sample from RHAc are presented in Figure 4.3.

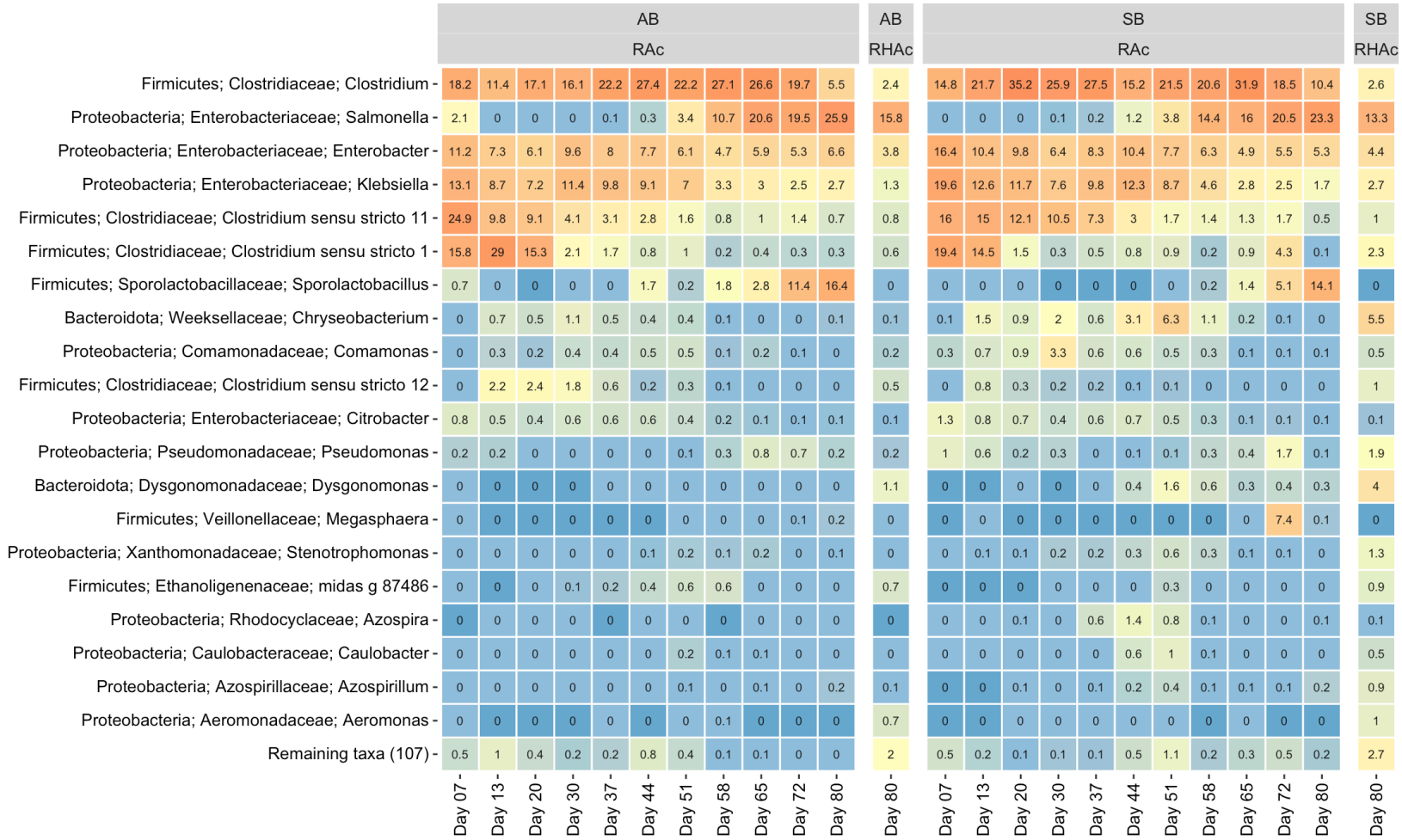


Figure 4.3 – Heatmap of relative abundance (%) of 20 domain genera during the operational period for both adhered (AB) and suspended (SB) biomass of the strictly acidogenic reactor (RAc) and final sample of the hydrolytic-acidogenic reactor (RHAc).

To study the beta-diversity, principal coordinate analysis (PCoA) with Bray-Curtis dissimilarity (Figure 4.4) was conducted and revealed that the microbial community evolved gradually throughout the operational period and that adhered and suspended biomass did not differ significantly from each other. The gradient representing the weeks during which the sample was collected, clearly represents the changes in diversity among samples. The first axis (PCo1) represents the most significant changes and correlates with temporal samples, which differ from each other monotonically, except for weeks 6 to 8 (from day 44 to 58).

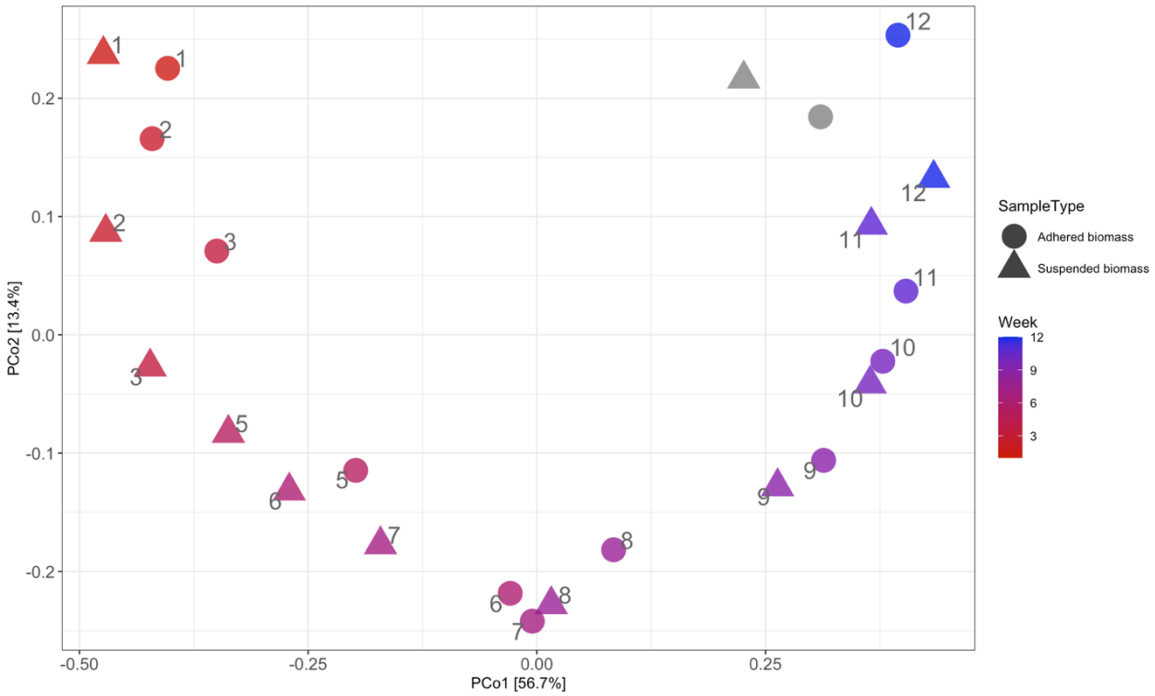


Figure 4.4 – Principal coordinate analysis with Bray-Curtis distance of adhered and suspended samples from the strictly acidogenic reactor (RAc) throughout the operational period and the final sample from the hydrolytic-acidogenic reactor (RHAc). Samples in grey are the final adhered and suspended biomass from hydrolytic-acidogenic reactor (RHAc).

The microbial community of RHAc was more diverse, compared to the RAc, even though both reactors had the same dominant bacterial groups. RHAc allowed the development of a hydrolytic community and other syntrophic groups due to a more substrate-complex environment and the presence of another stage of the AD process. In the RAc, abundant genera (> 1 % relative read abundance) were affiliated to *Proteobacteria* and *Firmicutes* phyla, while in RHAc, the *Bacteroidota* phylum was also significantly present. The genera evenness was higher in the RHAc, especially in the suspended biomass, contributing to a more balanced and diverse microbial community, which is indicative of a stable ecosystem (Castellano-Hinojosa et al., 2018). However,

a presumed higher hydrolytic activity during starch fermentation did not contribute to any greater cometabolic degradation of TBBPA. In fact, RAc had less variation and higher efficiency on TBBPA and organic matter removal (Table 4.1).

Fermentative bacteria affiliating with the *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Spirochaetes* phyla have been associated with TBBPA bioconversion (Iasur-Kruh et al., 2010; Lefevre et al., 2016; Yang et al., 2018; Xie et al., 2018; Lin et al., 2020). *Proteobacteria* and *Firmicutes* were the main phyla identified in the present study. Bacterial identification indicates that there is a predominance of the *Proteobacteria* phylum on TBBPA bioconversion (Xie et al., 2018; Wang et al., 2021 b). In addition, Gram-positive bacteria affiliated with the genera *Bacilli*, *Clostridia* and *Rhodococcus* have been extensively associated with TBBPA degradation in previous studies (Lefevre et al., 2016; Peng et al., 2017; Lin et al., 2020).

According to Lefevre et al. (2016), repeated exposure to high concentrations of TBBPA did not cause significant temporal shifts in the methanogenic community. However, other studies stated that besides the TBBPA's significant role in the changes of microbial community structure and composition (Iasur-Kruh et al., 2010; Xie et al., 2018; Chen et al., 2019), the pollutant is an intracellular metabolism disruptor (Wang et al., 2019). Moreover, Gram-positive bacteria rather than Gram-negative bacteria are more likely to be inhibited by TBBPA exposure as Gram-negative bacterial phyla have an outer membrane which confers increased resistance to hydrophobic antibiotics (Harrop, 1989; Xie et al., 2018). However, in all above-mentioned studies, TBBPA had been applied in concentrations higher than in this study and its environmental occurrence, from 2.5 to 50 mg L^{-1}. In the present study, even though the microbial community suffered from changes over long-term and continuous exposure to TBBPA under acidogenic conditions, the main genera of the microbiome stayed similar.

Potential TBBPA degraders and correlation factors

A Pearson's correlation plot for TBBPA and carbohydrate removal, OAs production, and the main bacterial genera in the RAc was made to identify statistical correlation to the main bacterial genera in the RAc (Figure 4.5). *Enterobacter* and *Clostridium* were the only two genera strongly and positively correlated to TBPPA biodegradation, with Pearson's coefficient of 0.72 and 0.69, respectively. This suggests that in the diverse acidogenic microbiome, these two genera may be the key players related to the cometabolic degradation of TBBPA at environmentally relevant

concentrations. The bioconversion of the pollutant may be achieved by strains from these genera as they share common enzymatic activities in the metabolic pathways for glucose acidification into OAs and the production of biohydrogen, even though they belong to different phyla.

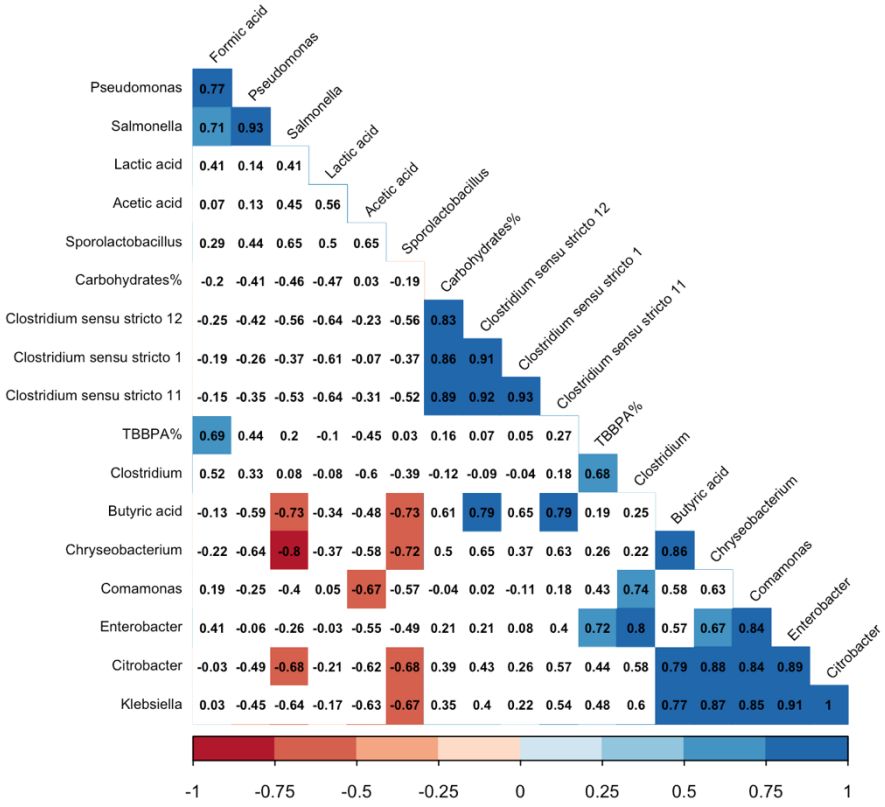


Figure 4.5 – Pearson's correlation plot for TBBPA and carbohydrates removal, organic acids production, and the main bacterial genera in the strictly acidogenic reactor (RAc).

A specific acidification route to organic acids may not be of importance on TBBPA cometabolic degradation as no significant correlation between TBBPA removal and any quantified organic acid production was observed. The cometabolic mechanism of TBBPA bioconversion may be affected by subproducts of acidogenesis acting as electron donors and providing the energy needed during reductive dehalorespiration, in which TBBPA is the electron acceptor (Fetzner, 1995; Zhang et al., 2005). BioH_2 as electron donor may be produced directly by *Enterobacter* and *Clostridium* organisms, but also provided via the interspecies hydrogen/formate transfer process from other hydrogen producers. In fact, BioH_2 and formate have been detected in the RAc and RHAc headspace and bulk, respectively. These substances are highly energetic electron donors with rapid diffusion and are involved in interspecies electron transfer within AD microbiomes

(Stams and Plugge, 2009). Therefore, besides the genera correlated to TBBPA cometabolism, other organisms may contribute to its degradation via interspecies association. Both *Clostridium* spp. and *Enterobacter* spp. have been associated with the degradation of other OMPs such as antibiotics (amoxicillin, tetracyclines, sulfadiazine, and sulfonamides), aromatic hydrocarbons (toluene, phenanthrene, and pyrene), anti-inflammatory drugs (diclofenac), and linear alkylbenzene sulfonate (LAS) (Yang et al., 2015; Aissaoui et al., 2017; Wang et al., 2018; Granatto et al., 2019; Yang et al., 2020).

This is the first study to bring statistical correlations on TBBPA biodegradation by *Clostridium* spp. and *Enterobacter* spp. during the fermentation of glucose and further conversion into organic acids. Even though these results point to a specific stage of the anaerobic process in which TBBPA is bioconverted and to the possible candidates responsible for the pollutant's breakdown at environmentally relevant concentrations, the enzymatic mechanism by which this conversion occurs is still unexplored.

Enrichment of TBBPA degraders from the mixed microbial community

At the end of the operational period, a biomass sample from the RAc was enriched over time in batch experiments with TBBPA concentrations ranging from 0.05 $mg \cdot L^{-1}$ to 5 $mg \cdot L^{-1}$ to evaluate the effect of unrealistic concentrations of TBBPA on the microbial community. The heat map of relative abundance (%) of 12 domain genera over each concentration is presented in Figure 4.6.

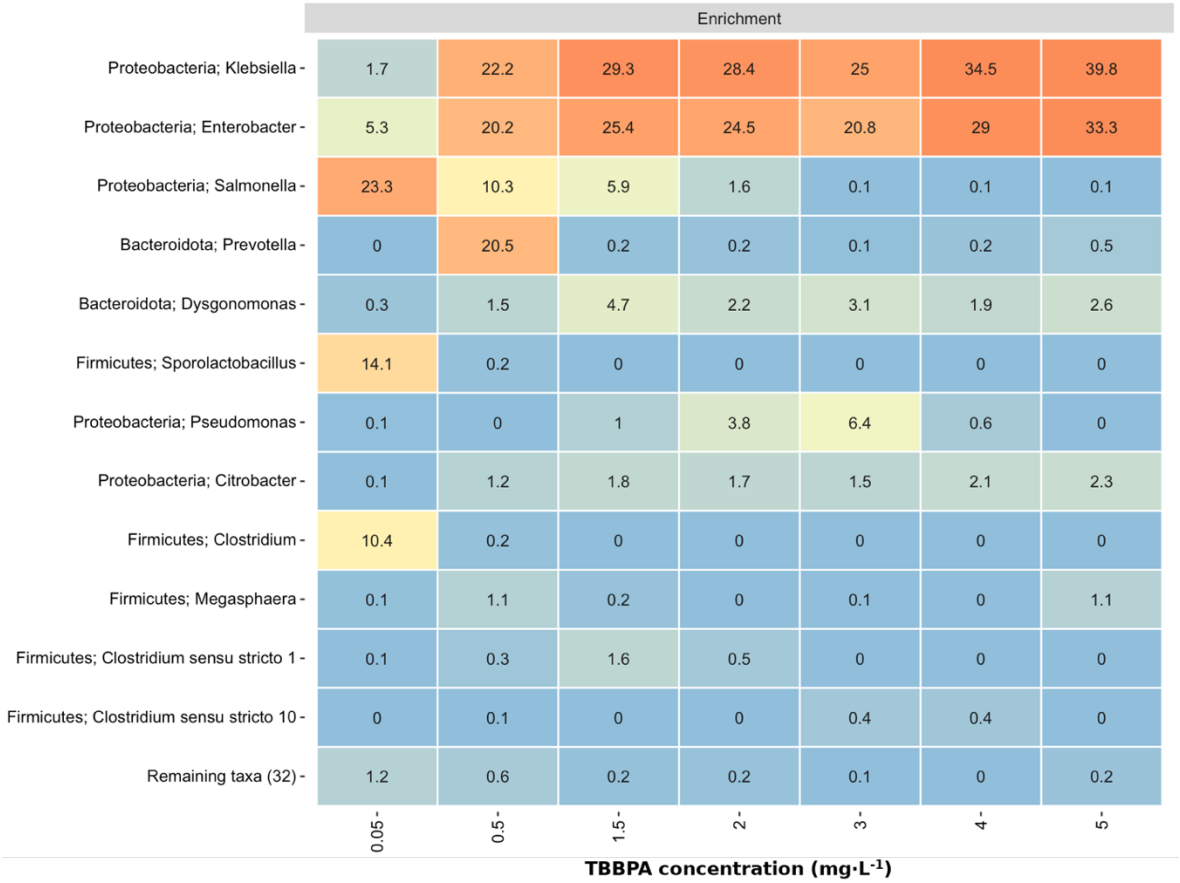


Figure 4.6 – Heatmap of relative abundance (%) of 12 domain genera during the enrichment process for each tested concentration of TBBPA.

The initial and final concentrations of TBBPA were quantified for each enrichment cycle, and the biodegradation efficiency was 98.9 ± 0.8 %. For the final enriched culture, TBBPA biodegradation reached 98.8 % in 4 hours and the stationary-phase for $BioH_2$ in 9 hours. The OAs detected were formic and acetic acids only. At environmentally relevant concentrations, *Clostridium* spp. was positively correlated to TBBPA degradation along with *Enterobacter* spp. However, when the concentration increased to 0.5 mg L^{-1}, this genus decreased its relative abundance from 10.4 % to 0.2 %. This corroborates the higher inhibition by TBBPA exposure to Gram-positive rather than Gram-negative bacteria (Xie et al., 2018). Conversely, by increasing TBBPA concentration up to 5 mg L^{-1}, *Enterobacter* spp. and *Klebsiella* spp. increased in relative abundance from 5.3 and 1.7 % to 33.3 and 39.8 %, respectively. Multiple species of the *Enterobacter* genus and the *Klebsiella* genus, from the MiDAS field guide database (Nierychlo et al., 2020), have a similarity of ≥98 %, which demonstrates that, phylogenetically, these organisms are closely related.

In the RAc, both *Salmonella* spp. and *Sporolactobacillus* spp. increased in relative abundance over time when subjected to a TBBPA concentration of 50 μg L^{-1}, but were negatively affected by higher concentrations. The *Prevotella* and *Pseudomonas* genera increased in relative abundance until reaching a TBBPA concentration of 0.5 and 3 mg L^{-1}, respectively. However, higher concentrations did not favour these organisms.

High concentrations of TBBPA may induce adaptations and microbial growth of specific strains, leading to metabolic routes that would not otherwise occur. As a consequence, conclusions in the direction of transformation products, co-metabolic preferences, microbial efficiency, and gene expression may be different in natural environments (Ghattas et al., 2017). The present study suggests that *Clostridium* spp. is a strong candidate for TBBPA degradation at environmentally relevant conditions by directly or indirectly (interspecies interaction) enabling the pollutant's removal. However, concentrations of 0.5 mg·L^{-1} or higher are detrimental to those organisms. For the *Enterobacter* spp. group, its positive correlation to TBBPA biodegradation in the RAc was further confirmed by the enrichment of these organisms under high concentrations of TBBPA and their degradation capacity.

Conclusion

The degradation of TBBPA during the anaerobic digestion process at environmentally relevant concentrations takes place concomitantly to pyruvate formation and further transformations toward short-chain fatty acids from glucose by non-specific groups of acidogenic bacteria. The specific hydrolytic activity is a negligible step in the bioconversion process of this pollutant, and the bioreactors' efficiency in relation to converting TBBPA is inversely proportional to the complexity of the wastewater. Moreover, hydrolysis had no influence on enhancing the kinetics of TBBPA bioconversion. During long-term exposure to TBBPA at concentrations close to its occurrences in WWTP, *Enterobacter* and *Clostridium* were the only two genera correlating to TBBPA biodegradation. The results of this study also highlight the importance of OMP degradation studies under realistic concentrations, as *Clostridium* spp. is a strong candidate for TBBPA degradation, but concentrations higher than 0.5 mg·L^{-1} are detrimental to those organisms.

Credit Author Statement

W.V. Macêdo: Conceptualization, Methodology, Data curation, Formal analysis, Investigation, Writing – original draft, Review, and Editing; **J. P. Poulsen**: Conceptualization, Methodology, Data curation, Writing – original draft, Review, and Editing; **G. H. D. Oliveira**: Conceptualization, Methodology, Data curation, Writing – original draft, Review, and Editing; **J. L. Nielsen**: Conceptualization, Methodology, Resources, Writing, Review, Editing, Supervision, and Funding acquisition; **M. Zaiat**: Conceptualization, Methodology, Resources, Writing, Review, Editing, Visualization, Supervision, Project administration, and Funding acquisition.

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Chapter 5

Proteogenomics identification of TBBPA degraders in anaerobic bioreactor

Abstract. Tetrabromobisphenol A (TBBPA) is the most used flame retardant worldwide and has become a threat to aquatic ecosystems. Previous research into the degradation of this micropollutant in anaerobic bioreactors has suggested several identities of putative TBBPA degraders. However, the organisms actively degrading TBBPA under *in situ* conditions have so far not been identified. Protein-stable isotope probing (protein-SIP) has become a cutting-edge technique in microbial ecology for enabling the link between identity and function under *in situ* conditions. Therefore, it was hypothesized that combining protein-based stable isotope probing with metagenomics could be used to identify and provide genomic insight into the TBBPA-degrading organisms. The identified ^{13}C-labelled peptides were found to belong to organisms affiliated to *Phytobacter*, *Clostridium*, *Sporolactobacillus*, and *Klebsilla* genera. The functional classification of identified labelled peptides revealed that TBBPA is not only transformed by cometabolic reactions, but also assimilated into the biomass. By application of the proteogenomics with labelled micropollutants (protein-SIP) and metagenome-assembled genomes, it was possible to extend the current perspective of the diversity of TBBPA degraders in wastewater and predict putative TBBPA degradation pathways. The study provides a link to the active TBBPA degraders and which organisms to favor for optimized biodegradation.

Keywords. Tetrabromobisphenol A; protein-SIP; metagenome-assembled genome; biodegradation; proteogenomics

Introduction

Organic micropollutants (OMPs) are chemical substances that occur at extremely low concentrations (μg – ng·L^{-1} range) and frequently detected in aquatic environments. These chemicals threaten both human health and the aquatic ecosystems due to their adverse ecotoxicological effects, their recalcitrant properties and ability to bioaccumulate in the food chain. OMPs derive from substances used in industry as pharmaceuticals, personal care products, antibiotics, and industrial products, such as flame retardants. Among the brominated flame

retardants, tetrabromobisphenol A (TBBPA) is the most used worldwide, and it is applied to plastics, ceramics, building materials, electronics, and epoxy in order to meet fire safety requirements. It has also been quantified in water bodies in concentrations of approximately 5 $\mu g \cdot L^{-1}$ (Yang et al., 2012; Macêdo et al., 2021). European legislation follows a precautionary principle and has introduced threshold limits for drinking water (not to exceed 100 $ng \cdot L^{-1}$) for several OMPs. TBBPA, for example, has been listed as a restricted substance by the European Union's Restriction of Hazardous Substances Directive (Xu et al., 2021). Effluents from municipal and industrial wastewater treatment plants (WWTPs) are the main source for OMPs entering the aquatic ecosystem, as most technologies for wastewater treatment are inefficient for removing the more recalcitrant OMPs (Ghattas et al., 2016).

Microbial removal techniques constitute a preferred way to remove organic pollutants from wastewaters, although only a very superficial understanding of the biodegradation mechanisms is available. Knowledge of the microbial communities performing natural attenuation of contaminated environments is crucial for optimizing wastewater treatment technologies. Even though the biodegradation of TBBPA has been investigated over the past 20 years (Macêdo et al., 2021), the current knowledge of its bioconversion is still far from being transferable to wastewater treatment biotechnologies. The anaerobic digestion (AD) of TBBPA and the organisms acting on its degradation remain unclear. One of the largest obstacles regarding studies on TBBPA biodegradation relates to the fact that most of the studies have been conducted using culture-dependent approaches and in unrealistic concentrations (in the range of $mg \cdot L^{-1}$), compared to its occurrence in wastewaters (Macêdo et al., 2021). Even though the findings from such approaches may shed light on TBBPA biodegradation, they also induce microbial growth of specific strains, gene expressions, and bias on metabolic pathways that would not occur otherwise.

Recent studies based on correlations between relative microbial composition and TBBPA removal over long-term exposure at environmentally relevant concentrations have suggested that TBBPA degradation in anaerobic biosystems occurs during glycolysis and further fermentation into fatty acids by *Enterobacter* spp. and *Clostridium* spp. (Macêdo et al., 2021b). However, the role of acidogenic microorganisms in TBBPA biodegradation was proposed from statistical evidence, and it was not experimentally confirmed. Additionally, in experiments conducted with such low concentration of the pollutant (in the range of ng or $\mu g \cdot L^{-1}$), all taxa involved in the

degradation process are likely not identified through such multivariate statistical correlation analyses.

The potential concentration-dependent bias used in culture-dependent approaches can be overcome by applying a combination of bioengineering process performance and cutting-edge metagenomic and metaproteomic techniques. Omics approaches allow for deeper insights into microbial ecology regarding structure and potential function. However, by using stable isotope probing techniques, it is possible to link the metabolic activity directly to distinct organisms (Jehmlich et al., 2016).

Protein stable isotope probing (protein-SIP) is a technique in which the assimilation of labelled target substrates is detected in the peptide level by state-of-the-art mass spectrometry. A microbial population is spiked with a labelled substrate (stable isotopes), which are assimilated by distinct populations actively degrading those compounds (Vogt et al., 2016). The peptides identified are assigned to phylotypes expressing those newly synthesized proteins by combining the metaproteomic data with the metagenome of the microbial community, enabling a direct link between the identity of the organisms and their metabolic functions (Jehmlich et al., 2016). Additionally, protein-SIP allows for the identification of food webs by quantifiying incorporation of the labelled substrate in a time-lapsed experimental design. From the mass spectrometry data, three important types of information can be retrieved: 1) the relative isotope abundance (RIA), which describes the percentage of labelled atoms in a peptide, reflecting the proportion of labelled substrate that was assimilated, 2) the labelling ratio (LR), which is the ratio of labelled to natural (unlabeled) peptides, reflecting the relative synthesis and degradation rate of individual proteins, and 3) the shape of the isotopic distribution, which indicates direct metabolization of isotopically labelled substrates or cross-feeding patterns (von Bergen et al., 2013; Jehmlich et al., 2016).

In cometabolic degradation of micropollutants, the greatest challenge is to obtain significant labelling when applying environmentally relevant concentrations of the pollutant. However, an accurate and sensitive quantification of incorporation of the labelled component and the combined physiological information from the protein-SIP approach allow to track carbon fluxes within the studied consortia (von Bergen et al., 2013; Jehmlich et al., 2016).

The vast majority of the protein-SIP studies (Taubert et al., 2012; Morris et al., 2012; Mosbaek et al., 2016; Kleiner et al., 2021; de Jonge et al., 2021) have applied labelled substrate as the main carbon source to track uptake and metabolic activity in microbiomes. To the best of our

knowledge, this is the first study to combine protein-SIP and metagenomic data to identify and elucidate functional prediction of microorganisms actively involved in the cometabolic degradation of a micropollutant at concentrations close to what has been detected in the environment. For this purpose, a complex microbial community from a continuous acidogenic bioreactor treating wastewater contaminated with TBBPA at 50 $\mu g \cdot L^{-1}$ was incubated in a batch experiment with $^{13}C_{12}$-TBBPA. A metagenome was used to identify the newly synthetized proteins involved in the degradation of TBBPA by performing protein-SIP. The time-course sampling was performed based on the degradation kinetics of the component.

Material and Methods

Microbial community and experimental set-up

Batch reactors were inoculated with suspended biomass collected from a continuous acidogenic bioreactor treating synthetic wastewater contaminated with TBBPA (influent concentration of 50 $\mu g \cdot L^{-1}$). The main carbon source in the bioreactor was glucose at an organic load rate (OLR) of 12 $kgCOD \cdot m^{-3} \cdot d^{-1}$, and it was operated for a hydraulic retention time (HRT) of 2.4 h. At the time of biomass sampling, the reactor had been operated for 85 days. The TBBPA removal efficiency in the system was greater than 99%.

For the protein-SIP, two batch experiments were set up with the biomass collected from the continuous reactor: 1) non-labelled TBBPA for kinetics degradation and as a control for labelled-peptides identification, and 2) $^{13}C_{12}$-TBBPA for protein-SIP analysis. The non-labelled batch experiment was performed in triplicate, the main carbon source was glucose, and TBBPA (labelled and non-labelled set-up) was skipped in the batches to a final concentration of 500 $\mu g \cdot L^{-1}$. Based on the degradation profile obtained in the non-labelled TBBPA batches and the first-order kinetics model fitting from the experimental data, the biomass from the labelled experiment was withdrawn when 50% (5.5 h), 75% (11 h), and 99.9% (120 h) of the added TBBPA had been biodegraded. Both experiments were operated under the same settings: F/M of 1, glucose as the main carbon source (5 $g \cdot L^{-1}$), temperature at 30°C, shaking at 200 rpm, and 5 $gVSS \cdot L^{-1}$. The initial pH of the batch runs was 8.0 to enable complete TBBPA solubilization.

Chemicals and analytical methods

The suspended solids (2540-E) and pH (4500-H + B) were measured in accordance with the Standard Methods (APHA, 2005). Tetrabromobisphenol A (TBBPA, 4,40-Isopropylidenebis (2,6-dibromophenol), 97% purity, CAS 79-94-7) (Sigma-Aldrich), and all other chemicals used in feed and nutrient solutions were at least of analytical grade. Labelled TBBPA ($^{13}C_{12}$ TBBPA) was purchased from Wellington Laboratories (Guelph, Canada). TBBPA quantification was performed by dispersive liquid-liquid microextraction (DLLME), followed by liquid chromatography-tandem mass spectrometry (LC–ESI–MS/MS) analysis. An Agilent Technologies 1260 Infinity chromatograph equipped with an Agilent Technologies Poroshell 120 EC-C18 column (50 mm × 3.0 mm; 2.7 μm) was used for the chromatographic separation. Tandem mass spectrometric analysis was performed using a 5500 QTRAP hybrid mass spectrometer (ABSciex) equipped with a TurboV^{TM} ion source, operated in the negative electrospray mode (ESI-). Details on the sample preparation, extraction procedure, analytical conditions, and ESI-MS parameters have been described elsewhere (Macêdo et al., 2020). Samples were collected, filtered in combi syringe filters with a coarse glass fiber prefilter and a small-pore membrane as the main filter (polytetrafluoroethylene) (1.0/0.20 μm), and analyzed on the same day. The organic acids (lactic, formic, acetic, propionic, butyric, isobutyric, valeric, and isovaleric) were quantified by a high-performance liquid chromatograph (HPLC, Shimadzu®) equipped with a UV-diode array detector (SDP-M10 AVP), refraction index detector (RID-10A), and an Aminex HPX-87H column (300 mm, 7.8 mm, BioRad®) (Penteado et al., 2013).

Degradation kinetics

The time series samples were collected each hour, until TBBPA reached concentrations below the quantification limit (< 0.1 μg·L^{-1}), extracted, and analysed by LC-ESI-MS/MS. A first-order kinetic model with a residual concentration (Camargo et al., 2002) was adjusted to the experimental data (Equation 1).

$$C = C_R + (C_0 - C_R)e^{-k^{app}\,\theta} \quad \text{(Equation 1)}$$

C: the concentration in the bulk;

C_0: the concentration in the influent;

θ: the HRT;

C_R: the residual concentration when the reaction rate value is zero;

k^{app}: the apparent specific first-order kinetic constant, which considers the average biomass in volatile suspended solids ($gVSS \cdot L^{-1}$).

After fitting the model to the experimental data, the estimated parameters were used to predict the biomass collection points for the $^{13}C_{12}$-TBBPA batches.

Metagenome and bioinformatics

A metagenome was prepared, and DNA was extracted using FastDNA® SPIN®Kit for soil (MP Biomedicals), following manufacturer's recommendations. The metagenome preparation and sequencing were conducted by Novogene (United Kingdom). Usearch10 (Edgar, 2010) was used to remove PhiX, and the reads were trimmed for adaptors and filtered for a minimum phred score of 20 using cutadapt (Martin, 2011). The trimmed reads were assembled using SPAdes (v3.12.0) (Bankevich et al., 2012), applying k-mers of 21, 33, 55, and 77, and a minimum scaffold length of 1 kbp. To generate the files needed for the binning process in R, using the mmgenome2 package (Albertsen et al., 2013), a script from https://github.com/Kirk3gaard/misc_scripts/tree/master/prepare_data_for_mmgenome2 was used. In brief, the open reading frames in the metagenome were predicted using prodigal (Hyatt et al., 2010), essential genes were identified using HMMER (v.3.2.1) (http://hmmer.org/), and kaiju (Menzel et al., 2016) run in MEM mode, was used to taxonomically classify the contigs. Metagenome-assembled genomes (MAG) containing target genes for labelled peptides were extracted with the locator tool in mmgenome2 and manually curated. CheckM (Parks et al., 2015) was used for checking completeness and contamination, and the extracted MAGs were annotated using Prokka (Seeman et al., 2014). The functional annotation of the MAGs was performed through the MicroScope platform (Vallenet et al., 2019).

Protein extraction and protein-SIP analysis

Extraction of proteins from sludge matrix was carried out as previously described (Heyer et al., 2013) using TEAB (0.05 M TEAB buffer stock, 1.0 mg/L NaDOC, pH ≤ 8) as a resuspension buffer. After being extracted, the proteins undergo in-gel digestion, as described elsewhere (Shevchenko et al., 2007). The tryptic peptides were desalted and analysed by automated liquid chromatograph-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS), as described previously (Poulsen et al., 2021). For the identification of labelled proteins, a two-search strategy

was utilized. First an initial search against the NCBIprot database, using Mascot v.2.5 (Matrix Science, London, UK), with the peptide mass tolerance and MS/MS tolerance of ±1.2 Da and ±0.6 Da, respectively. Identified proteins from the survey search were exported from NCBI and merged with the metaproteome generated from the metagenome. The metaproteome was annotated using Prokka (Seemann, 2014). The main search was carried out in an Open MS pipeline (Sturm et al., 2008), where proteins/peptides were identified using OMSSA (Geer et al., 2004) with a precursor mass tolerance of 5 ppm, fragment mass tolerance of 0.01 Da, and a false discovery rate (FDR) of 1%. Metabolically active guilds were identified by mapping labelled and unlabelled protein identifications on the genome assembly. The relative isotope abundance (RIA) was determined using OpenMS and the MetaProSIP tool (Sachsenberg et al., 2015) with a minimum correlation of 0.7 and a mass window of 10 ppm.

Data availability

Metagenome data is available at the European Nucleotide Archive (ENA) under project accession number PRJEB49211. The mass spectrometry proteomics data have been deposited at ProteomeXchange Consortium (Vizcaíno et al., 2014) via the PRIDE partner repository with the data set identifier PXD030335.

Results

Degradation kinetics

The parameters of the adjusted kinetic model for the time-series profile of TBBPA are presented in Table 5.1. Based on the apparent specific first-order kinetic constant and in the residual concentration where the reaction rate value is zero, it was predicted that 50%, 75%, and 99.9% of the $^{13}C_{12}$-TBBPA degradation would be achieved after 5, 9, and 102 h, respectively. Therefore, the time-series samples were collected in the $^{13}C_{12}$-TBBPA-incubation at those points for protein-SIP analysis.

Table 5.1 – First-order kinetic expressions estimated for the TBBPA biodegradation. C_0 and C_r are in $\mu g \cdot L^{-1}$ and k^{app} in $h^{-1} \cdot g \cdot VSS^{-1}$.

Parameter	Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
C_r	5.21	5.825	-6.812	17.232
C_0	423.075	11.485	399.371	446.778
k^{app}	0.151	0.011	0.128	0.174

Metagenome analysis

The metagenome generated from the biomass yielded a grand total of 120,914,732 paired-end reads, which, when assembled, resulted in 93,723,561 bp divided into 15,420 different scaffolds. The size of the scaffolds varied from 984 to 361,219 bp, with an average scaffold size of 6,078 bp. The metagenome consisted of two different identified phyla: *Firmicutes* (17.2%) and *Proteobacteria* (15.0%). However, 67.9% of the scaffolds were not taxonomically classified. Figure 1 presents an overview on the taxonomic classification and distribution of the microbial community. The four most abundant families were the *Ruminococcaceae* (14.0%), *Enterobacteriacea* (7.9%), *Sporolactobacillaceae* (2.2%), and *Clostridiaceae* (0.9%). The genera *Ethanoligenes* (13.4%), *Phytobacter* (8.0%), *Klebsiella* (5.7%), *Sporolactobacillus* (2.2%), and *Clostridium* (0.9%), were the most abundant among the identified scaffolds.

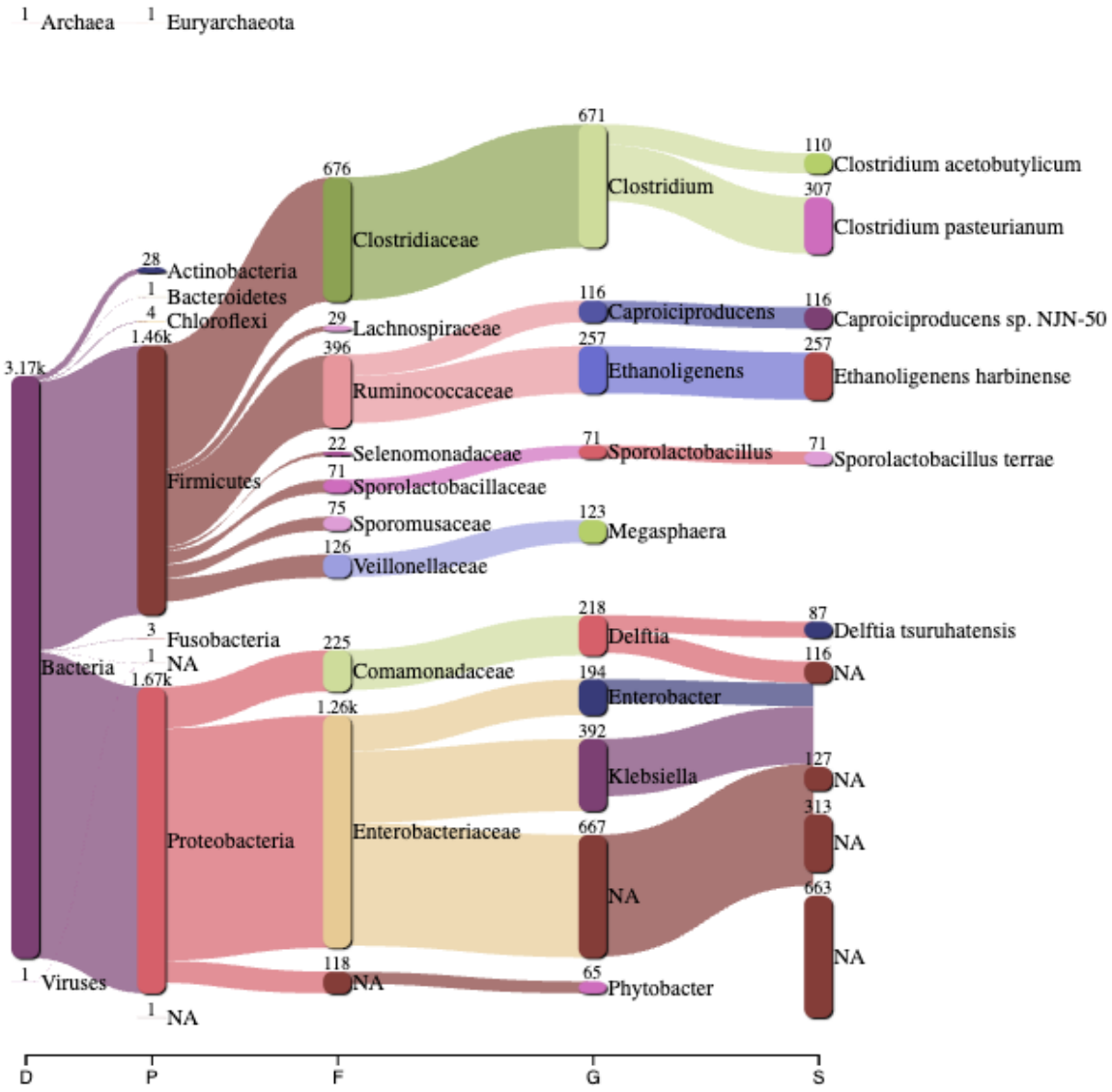

Figure 5.1 – Sankey diagram displaying the composition of the microbiome from a acidogenic bioreactor and incubated with ^{13}C-TBBPA.

Protein-SIP results

A total of 2774 peptides were identified across the four samples (blank, 50%-, 75%-, and 99.9%-degradation). Among all peptides, 10 different ones were found to have assimilated labelled carbon and the relative isotope abundance (RIA) ranged from 3.7 to 5. Most of the labelled peptides were found in sample 99.9%-degradation. The majority of the labelled peptides belonged to

organisms affiliated to the *Enterobacterceae* family, followed by the *Clostridiaceae*, and the *Sporolactobacillaceae* families. All labelled peptides were identified and mapped to the respective scaffold (Figure 5.2), which were divided into five different bins of the metagenome. The peptides were identified as part of carbon-carbon lyases, transporters, oxidoreductases, and structural proteins. The labelled peptides, protein description, RIA2 value, phylogenetic affiliations of the peptide, and the samples in which they were found are presented in Table 5.2.

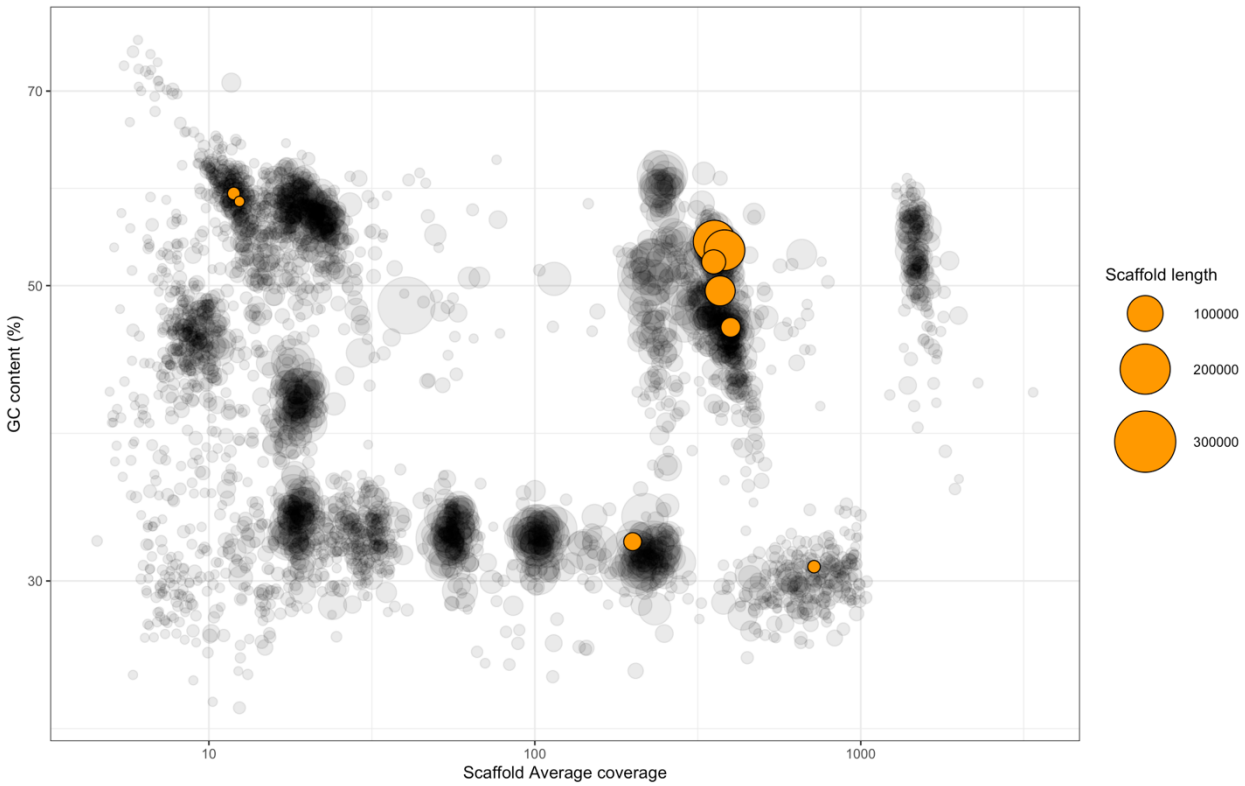

Figure 5.2 – Differential coverage binning of the combined metagenome, shown as scaffold coverage and GC content. The dot sizes indicate the scaffold length, and with a minimum scaffold length of 6000 bp. Colouring is according to the scalffold containing a template for the identified ^{13}C-labelled proteins.

Table 5.2 – Peptides for which an increase in isotopic incorporation of ^{13}C was observed in the degradation experiment.

Protein	Sample	Accession number	Peptide sequence	Protein description	RIA2	Phylogenetic affiliation	
						Family	Species
1	50%	KPKJAHNK_38736	SLVSEELTK	6-diphosphate aldolase	4.8	*Sporolactobacillaceae*	*Sporolactobacillus terrae*
2		KPKJAHNK_57630	LTSSPAAVSWGNNR	hypothetical protein	3.7	*Clostridiaceae*	*Clostridium pasteurianum*
3		WP_028114164.1	LASTWEGIQAAR	Transaldolase	4.1		*Klebsiella pseudopneumoniae*
4	75%	KPKJAHNK_28756	DAEQDIILQEMYDK	LPS-assembly lipoprotein LptE	0		*Phytobacter ursingii*
5	99.9%	KPKJAHNK_05461	AAFDQWVAK	Cytochrome bo3 quinol oxidase subunit 2	4.7		*Phytobacter ursingii*
6		PWF51883.1	AAFDQWVAK	Cytochrome o ubiquinol oxidase subunit II	5	*Enterobacterceae*	*Phytobacter ursingii*
7		KPKJAHNK_06149	QETGDNVTIR	Sulfate ABC transporter substrate-binding protein	5		*Phytobacter ursingii*
8		KPKJAHNK_18028	EFVESLETPR	6-Phosphogluconate Dehydrogenase	4.3		*Phytobacter ursingii*
9		KPKJAHNK_42614	PVINHNLSAINANR	Flagellin	3	*Clostridiaceae*	*Clostridium pasteurianum*
10		WP_042394572.1	QETGDNVTIR	Sulfate ABC transporter substrate-binding protein	5	*Enterobacterceae*	*Klebsiella pseudopneumoniae*

Extraction of labelled metagenome-assembled genomes

A total of 5 MAGs (Bin 1-5), containing coding sequences for the labelled peptides, were extracted using differential coverage binning of the assembly (Figure 5.3). Quality control of the bins showed that Bin 5 had the highest completeness of 95.5%, and Bin 2 and 3 had the lowest contamination of 8.07% and 0.00%, respectively (Table 5.3).

Table 5.3 – Information on the five labelled MAGs.

Bin name	Scaffolds	Length (bp)	Labelled peptides	Completeness (%)	Contamination (%)
Bin 1	258	4,952,189	1	93.1	122.4
Bin 2	424	3,611,852	1	70.5	8.1
Bin 3	868	5,136,113	2	21.5	0.0
Bin 4	254	7,940,176	4	90.5	185.3
Bin 5	130	4,737,853	1	95.5	148.6

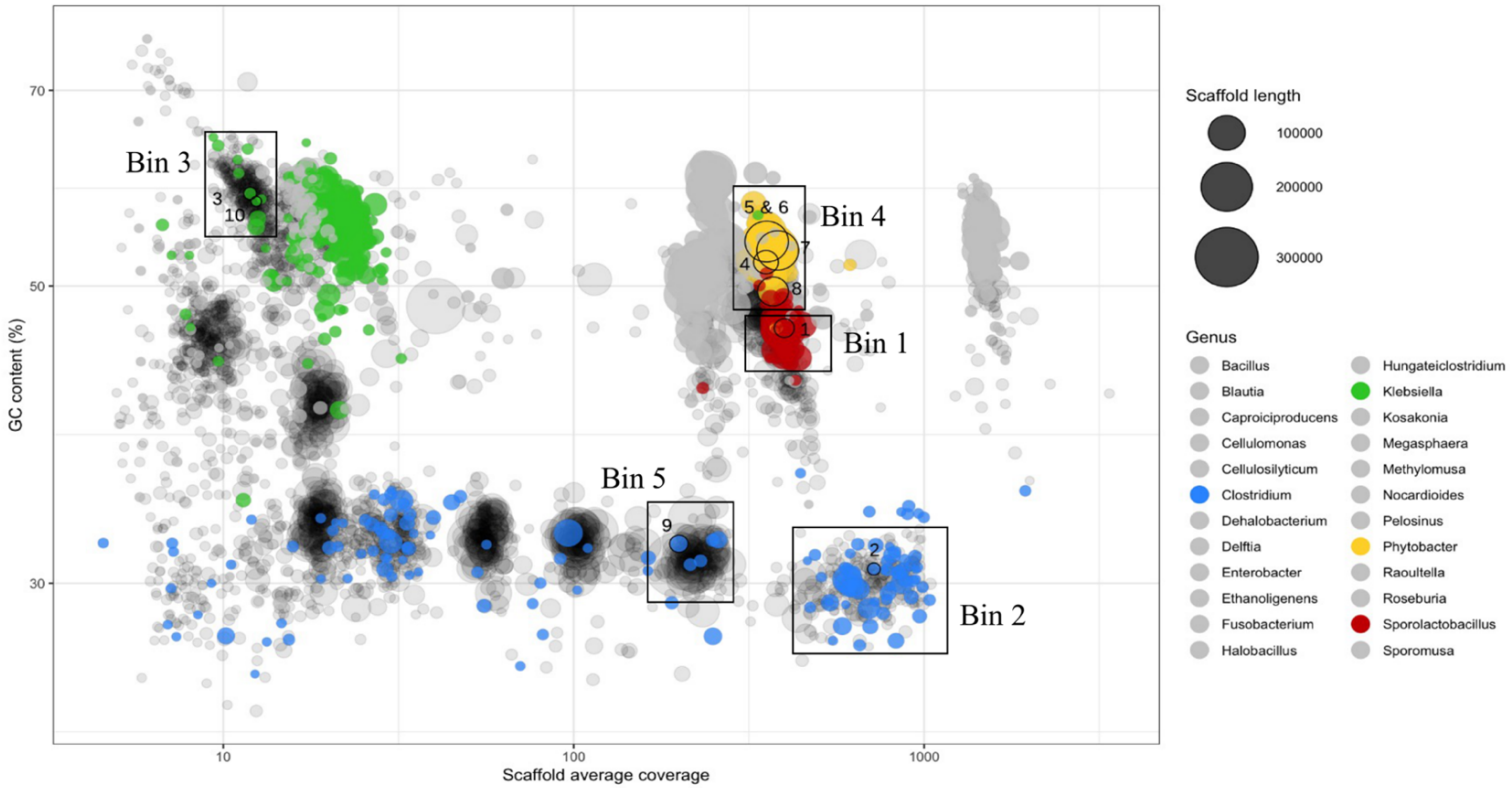

Figure 5.3 – Differential coverage binning of the combined metagenome, shown as scaffold coverage and GC content. The dot sizes indicate the scaffold length, and with a minimum scaffold length of 6000 bp. a) Colouring is according to taxonomy (genus level) and only the genera containing a template for the identified ^{13}C-labelled proteins (numerically identified from 1 to 10) are coloured. The scaffolds containing templates for labelled proteins are depicted in boxes numerically identified from Bin 1 to 5.z

One of the five bins (bin 2) fulfils the requirements for a medium quality MAG (completeness ≥50% and contamination <10%), and three of the bins (bins 1, 4, and 5) all had a completeness above 90%, even though they had high contamination. The last bin (bin 3) had a completeness of 21.6%. Three of the bins were classified to the phylum *Firmicutes* (bins 1, 2, and 5), and two bins were classified to *Proteobacteria* (bins 3 and 4). These bins included representatives of *Sporolactobacillus terrae* (bin 1), *Clostridium pasteurianum* (bin 2), *Klebisiella pseudopneumoniae* (bin 3), *Phytobacter ursingii* (bin 4), and *Clostridium pasteurianum* (bin 5) species. The bins contained one to four labelled proteins each, including dehydrogenase, 6-disphosphate aldolase, ABC transporters, and electron carriers (Table 5.2).

After manual curation to remove the contaminants, a predicted proteome for each one of the extracted metagenome-assembled genomes (bins 1 to 5) was generated. From the predicted proteomes, putative proteins involved in TBBPA degradation were identified and are presented in Table 5.4. Additionally, an automatic functional annotation of the genomes was assessed using the MicroScope® software package. From the MicroScope® functional annotation, many genes coding for proteins involved in the degradation of aromatic and halogenated compounds were identified. Bins 1 and 4 contained genes coding for the metabolism of 3-chlorocatechol, phenol, and catechol degradation, among many other aromatic compounds. In addition, these bins contained genes coding enzymes related to dehalorespiration (NADH to cytochrome bd/bo) as well as hydrogen production. All bins contained genes related to fermentative metabolism, which would potentially carry out the cometabolism of TBBPA in anaerobic bioreactors and explain the relatedness to acidogenesis (Macêdo et al., 2021).

Discussion

Protein-SIP

Several peptides showed clear isotopic labelling profiles, both in the relative isotope abundances (RIA) and the shape of the isotopic distribution. The labelling of proteins by feeding a complex microbial community with labelled compounds in the concentration range relevant for micropollutants occurrence was a challenge addressed in the present study. Already in the first sample, after 5 h of incubation, labelled proteins were identified with an RIA of approximately 5%, which did not change significantly with longer incubations (from 5.5 to 120 h). The constant and

time-independent RIA values indicate that labelled TBBPA was assimilated as a general carbon source as part of a cometabolic strategy together with glucose conversion at a 4:96 ratio. The isotopic distribution of the identified peptides followed a symmetrically normal distribution, revealing that the peptides undergo direct metabolization of isotopically labelled substrates and likely not through cross-feeding patterns (von Bergen et al., 2013; Jehmlich et al., 2016).

It should be noted that one of the most known pathways on TBBPA degradation is its complete debromination to BPA followed by further degradation of BPA (Macêdo et al., 2019). In this experiment, the TBBPA chemical structure had ^{13}C-labelled aromatic rings, which can suggest that the labelled proteins were associated to BPA-degraders rather than TBBPA-degraders. This hypothesis is not considered as the complex microbial community incubated in the present study was collected from a continuous acidogenic bioreactor treating wastewater contaminated with TBBPA at 50 $\mu g \cdot L^{-1}$ over 3 months. Additionally, in a differential proteomics experiment performed by the authors (unpublished data), based on monitoring free-bromide concentration throughout a similar incubation and the theoretical concentration that would be yielded by the initial TBBPA concentration, it has been seen that when TBBPA degradation reaches 99%, the debromination of TBBPA only reaches 50% of what would be expected when TBBPA is fully degraded solely by reductive debromination followed by BPA accumulation. This suggests that the cleavage of the aromatic rings may occur prior to full debromination. Lastly, to our best knowledge and in this scenario, there is no other state-of-art technique that would result in more reliable labelling of proteins rather than protein-SIP with labelled carbon in the aromatic rings since no organism uses bromide for cellular growth.

Labelled peptides were identified in all samples, even with the F/M ratio of the main carbon source (glucose) being 10,000-fold higher, compared to the F/M ratio of the labelled micropollutant. This demonstrates the high precision and accuracy of the protein-SIP approach and its ability to investigate the cometabolism of micropollutants at environmentally relevant levels.

Several published protein-SIP studies have identified labelled peptides with a higher RIA ($\geq$ 20%) and labeling ratio (LR) ($\geq$ 10%) values (Taubert et al., 2012; Morris et al., 2012; Vogt et al., 2016; Mosbaek et al., 2016; de Jonge et al., 2021). However, this is the first study in which the labelled compound was used in the $\mu g \cdot L^{-1}$ as part of a cometabolism together with glucose as the main energy and carbon source. Unrealistically high concentrations of micropollutants ($mg \cdot L^{-1}$), compared to those occurring in natural systems, can induce specific gene expression and microbial

growth of particular strains. By using a culture-independent protein-SIP approach on biomass adjusted to grow on concentrations close to its environmental occurrence, the knowledge on the active TBBPA-degrading organisms would reflect biotreatment technologies in engineered systems.

Studies using similar technological approaches on anaerobic degradation of other aromatic pollutants such as benzene, toluene, ethylbenzene, and xylene have identified degraders affiliating to the same phyla as for the TBBPA, namely *Firmicutes* and *Proteobacteria* (Taubert et al., 2012; Vogt et al., 2016). The functional classification of the identified proteins was mainly hypothetical proteins, transport and binding, regulation, and ATP synthesis proteins, which aligns well with the results of this study.

Phylogenetic and functional annotation of identified proteins

The taxonomic information from the 5 extracted bins containing target genes for the isotopically labelled peptides allowed the identification of five active TBBPA degraders: *Sporolactobacillus terrae* (bin 1)*, Clostridium* spp. (putative *C. pasteurianum*) (bin 2), *Klebsiella pseudopneumoniae* (bin 3)*, Phytobacter ursingii* (bin 4), and *Clostridium* spp. (putative *C. pasteurianum*) (bin 5). Seven of the ten labelled peptides were affiliated to the *Enterobacterceae* family, two to the *Clostridiaceae*, and the last one to the *Sporolactobacilliceae* families, which are part of the most abundant acidogenic bacteria in anaerobic biosystems (Hung et al., 2011; Ferraz et al., 2020; Park et al., 2021). To the best of our knowledge, this is the first study in which the above-mentioned species have been associated to degradation of micropollutants, except for *Klebsiella* spp., which has previously been associated with the degradation of aromatic compounds. The facultatively anaerobic *Klebsiella* genus (*Enterobacterceae* family) has been related to the degradation of linear alkylbenzene sulfonate (LAS) (Pandey et al., 2020), polycycli aromatic hydrocarbons (Xu et al., 2019), anti-inflammatory drug diclofenac sodium (Sharma et al., 2020), and aliphatic and aromatic hydrocarbons from crude oil (Chamkha et al., 2011). Like *Phytobacter* spp., these organisms also possess nitrification and denitrification properties. The genera *Phytobacter,* which is also affiliated to the *Enterobacterceae* family, has previously been described as a potential fermentative hydrogen producer in wastewater containing high concentrations of heavy metals (Cho and Lee, 2011), as a denitrifier in membrane bioreactors (Choi et al., 2021), and

nitrogen-fixing organisms (Pillonetto et al., 2018). Both organisms have similar genomic information and possess nitrification and denitrification properties.

Organisms affiliated to the *Clostridium* genus are predominant biohydrogen producers and producers of carbohydrates active enzymes, associated with the degradation of recalcitrant polymers (Hung et al., 2011; Blair et al., 2021). The *Sporolactobacillus* genera are lactic acid producers and have been associated with the shift in fermentation products from H_2/acetate/butyrate to lactate/ethanol. In acidogenic systems, *Sporolactobacillus* and *Clostridium* have been reported as major H_2-producing genera (Ferraz et al., 2020; Park et al., 2021).

All organisms identified as active TBBPA degraders in this study are representatives of phyla associated to fermentative hydrogen production, which suggests that reductive dehalorespiration could be one of the primary mechanisms for TBBPA breakdown. In this process, $BioH_2$ and TBBPA are the electron donor and acceptor, respectively (Fetzner, 1998). When performing dehalorespiration, fermentative bacteria utilize several electron donors and acceptors in a respiratory system involving hydrogenases, reductive dehalogenases, and electron carriers mediating electron transfer between them with relaxed substrate specificity (Fetzner 1998; Macêdo et al., 2021). Analogously, chlorinated compound degradation by the sulfate-reducing bacterium *Desulfomonile tiedjei* has the capacity to use hydrogen or formate as an electron donor and 3-chlorobenzoate as a terminal electron acceptor in a respiratory process (Fetzner 1998).

Besides the metabolic dehalorespiration, bacteria affiliated to the *Enterobacterceae* family have been reported to perform a presumably cometabolic dehalogenation with no benefit for the organism via electron carriers of the respiratory electron-transport chains (Fetzner, 1998). Additionally, as another cometabolic process, reduction of tetrachloromethane was also observedin bacteria possessing the acetyl-CoA pathway (Egli et al., 1988; Fetzner, 1998), and by analogy, the same process could be involved in TBBPA-reductive debromination and further degradation.

Predicted proteomes from metagenome-assembled genomes (MAGs)

A predicted proteome for each one of the extracted metagenome-assembled genomes (bins 1 to 5) was generated as well as the automatic functional annotation via MicroScope. The relevant proteins and metabolic pathways for the cometabolic degradation of TBBPA, which are presented in Tables 5.2 and 5.4, suggest that several degradation pathways are mediated by enzymes with relaxed substrate specificity. Proteins such as hydrogenases, oxidoreductases, dehalogenases, and

electron transport carriers (ETC) mediating electron transfer have previously been related to the degradation of halogenated aromatic compounds (Fetzner, 1998), which were found in all of the five predicted proteomes. For example, oxidative dehalogenation is mediated by mono- or dioxygenases in cometabolic or metabolic reactions. Hydrolytic dehalogenation reactions occur by the substitutive dehalogenation catalyzed via halidohydrolases, and lastly, the reductive dehalogenation, which is the most commonly reported step in TBBPA degradation (Macêdo et al., 2021). Dehalogenases and electron carrier enzymes are responsible for the hydrolytic dehalogenation, and the process is carried out both as a cometabolic and a metabolic (respiratory) process (Hardman 1991; Haggbolm, 1992; Fetzner 1998). Monoxygenases and dioxygenases are essential enzymes for the hydroxylation and cleavage of aromatic ring structures (Haggblom, 1992; Heider and Fuchs, 1997), and oxidoreductase enzymes were present in the predicted proteomes of bins 1, 2, and 4.

From the metabolisms presented in Tables 5.2 and 5.4, the identified organisms could express different degradation routes of the pollutant. Additionally, organisms affiliated to bin 1 (*Sporolactobacillus terrae*) and bin 4 (*Phytobacter ursingii*) are suggested to be the best candidates for the primary degradation of TBBPA and likely responsible for the cleavage of the aromatic rings and for facilitating metabolic dehalorespiration via electron transfer, using TBBPA as electron acceptor.

Table 5.4 – Proteins from the predicted proteomes generated from the Bins 1 to 5 possibly related to TBBPA biodegradation.

Bin	Organism	Protein	Function	Reference
1	*Sporolactobacillus terrae*	Haloalkane dehalogenase	Catalyzes hydrolytic cleavage of carbon-halogen bonds in haloaromatic compounds	Keuning et al. (1985) Yokota et al. (1987)
		p-hydroxybenzoate hydroxylase transcriptional activator	Benzoate degradation; Degradation of aromatic compounds; Xenobiotics degradation	Spector and Massey (1972)
		4-hydroxybenzoate transporter PcaK	Transport activity of aromatic compounds	Pernstich et al. (2014)
		3-octaprenyl-4-hydroxybenzoate carboxy-lyase	Degradation of aromatic compounds	Baker et al. (2014)
		Benzoate 1,2-dioxygenase electron transfer component	Benzoate degradation; Degradation of aromatic compounds; Xenobiotics degradation	Reiner (1972)
		2-halobenzoate 1,2-dioxygenase small/large subunit	This protein is involved in the pathway benzoate degradation via CoA ligation, which is part of Xenobiotic degradation	Fetzner et al. (1992)
		2-haloacrylate reductase	The enzyme acts in the degradation pathway of unsaturated organohalogen compounds	Kurata et al. (2005)
		Chloramphenicol acetyltransferase 3	Antimicrobial resistance genes	Shaw and Brodsky (1967)
		Polyphenol oxidase	Benzene degradation	Cadieux et al. (2002)
		Phenolic acid decarboxylase subunit D	Degradation of aromatic compounds	Tschech and Fuchs (1989)
2	*Clostridium pasteurianum*	4-hydroxybenzoate transporter PcaK	Transport activity of aromatic compounds	Pernstich et al. (2014)

		Cytochrome c biogenesis protein Ccs1	Required during biogenesis of c-type cytochromes	Dreyfuss et al. (2002)
		p-hydroxybenzoic acid efflux pump subunit AaeA	Transport activity of aromatic compounds	Touchon et al. (2009)
3	*Klebsiella pseudopneumoniae*	Phenolic acid decarboxylase subunit D	Degradation of aromatic compounds	Tschech and Fuchs (1989)
4	*Phytobacter ursingii*	Formate dehydrogenase, nitrate-inducible, cytochrome b556(Fdn) subunit	Formate dehydrogenase allows the use of formate as major electron donor during anaerobic respiration	Jormakka et al. (2002)
		Aromatic amino acid exporter YddG	Probable efflux pump	Blattner et al. (1997)
		Cytochrome bd-II ubiquinol oxidase subunit 2	Energy metabolism; Electron transfer	Dassa et al. (1991)
		Cytochrome b561	Transmembrane electron carriers	Nakanishi et al. (2009)
		p-hydroxybenzoic acid efflux pump subunit AaeA	Transport activity of aromatic compounds	Touchon et al. (2009)
		Haloalkane dehalogenase	Catalyzes hydrolytic cleavage of carbon-halogen bonds in haloaromatic compounds	Scholtz et al. (1987)
		4-hydroxybenzoate transporter PcaK	Transport activity of aromatic compounds	Pernstich et al. (2014)

According to the several groups of proteins present in the predicted proteomes of the active TBBPA-degraders, the pollutant most likely undergoes multiple degradation pathways: 1) reductive debromination by dehalogenases, 2) cleavage of aromatic rings by reductases acting on carbon-carbon bonds, 3) carbon-hydrogen bonds oxidized by hydroxylases, 4) hydroxylation and cleavage of the aromatic rings by monoxygenases and dioxygenases, 5) dehalorespiration facilitated by electron transfer carriers and hydrogenases. Some of the possible degradation pathways performed by both *Sporolactobacillus* and *Phytobacter* organisms are illustrated in Figure 5.4.

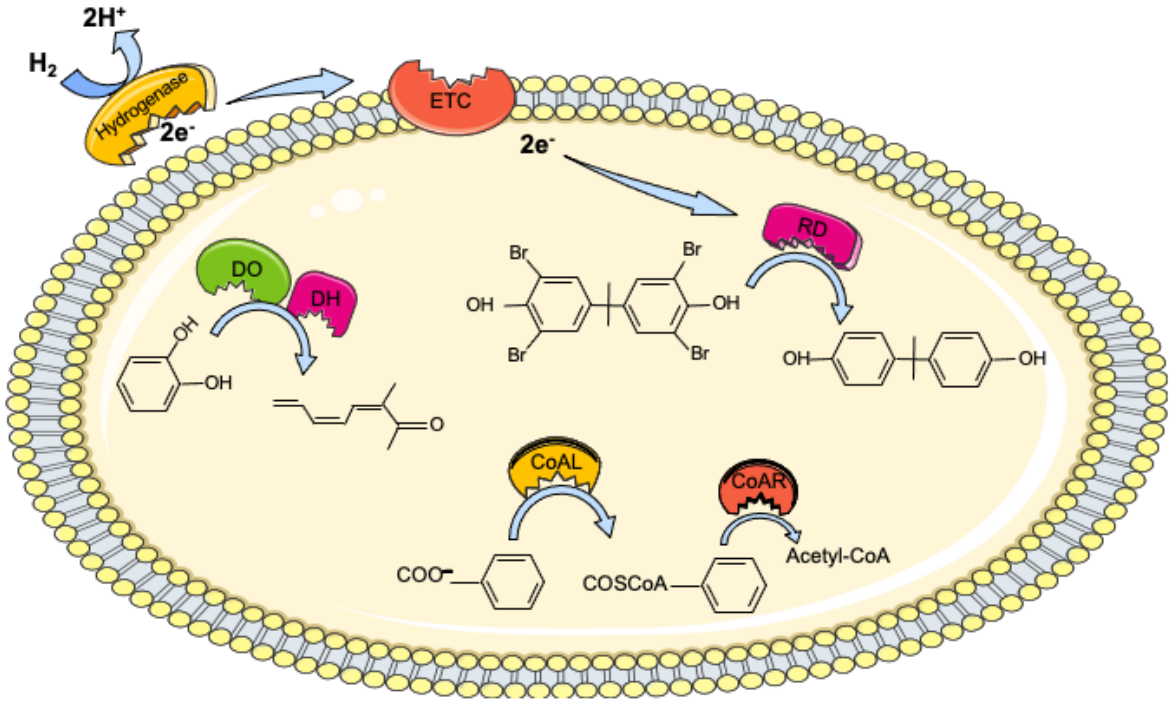

Figure 5.4 – Encoded enzymes from *Sporolactobacillus* and *Phytobacter* metagenome-assembled genomes possibly involved in TBBPA degradation. ETC: electron transfer carrier; RD: reductive dehalogenase; DO: dioxygenase; DH: dehydrogenase; CoAL: CoA ligase; CoAR: CoA reductase.

Conclusion

Microorganisms actively involved in the degradation of TBBPA were identified by a proteogenomic approach, using protein-based stable isotope probing (protein-SIP) and metagenomic binning. This approach allowed for the identification of organisms assimilating labelled carbon by a cometabolic degradation of the ^{13}C–TBBPA in environmentally relevant concentrations. The active degraders were affiliated to the genera *Sporolactobacillus*, *Phytobacter*, *Clostridium*, and *Klebsiella* within the *Firmicutes* and *Proteobacteria* phyla. Based on the extracted

metagenome-assembled genomes containing the reference sequences for the labelled proteins, it was predicted that both cometabolic pathways and respiratory (dehalorespiration) mechanisms are involved in the transformation of TBBPA. This study provides better insight into the identity of TBBPA-degrading organisms and potential degradation processes, thereby presenting a step forward for the understanding of how biosystems can be optimized for micropollutant removal.

General discussion and Conclusion

This work was motivated by the need of extending knowledge on how micropollutants are biodegraded in anaerobic reactors. Knowledge on TBBPA biodegradation would contribute to a better understanding of other flame retardants environmental fate and to the further optimization of wastewater treatment biotechnologies. The studies conducted throughout this doctoral thesis started with less specific questions (in which stage of the anaerobic digestion) to a more in-depth investigation (where exactly, who, and how) on the degradation process. Prior to evaluating the removal efficiency of TBBPA in bioreactors and understanding the microbiome involved in the process, we proposed a miniaturized procedure used to extract and quantify the analyte in domestic sewage, anaerobic sludge, and aquatic test-organism species, (*Daphnia magna* and *Chironomus sancticaroli*). The presented extraction and quantification method represents a simple and cost-effective tool to determine TBBPA environmental occurrence and biomagnification potential compared to conventional extraction methods. With these findings, we confirmed the first hypothesis presented in this thesis: Miniaturized liquid-liquid dispersive extraction is an efficient and more eco-friendly alternative to quantify TBBPA in complex matrices.

In sequence, two anaerobic bioreactors were operated with synthetic domestic sewage contaminated with TBBPA at environmentally relevant concentrations to shed light in which stage of the anaerobic digestion the degradation of the pollutant would occur. In this experiment, we confirmed the second hypothesis by bringing evidence that TBBPA was degraded during acidogenesis. Furthermore, we highlighted via TBBPA mass balance that, in continuous systems fed with TBBPA in the concentration range of $\mu g \cdot L^{-1}$, the adsorption of the pollutant accounts only for 1 to 3% of the influent TBBPA and that, in AD systems, the degradation of TBBPA is not limited to reductive debromination.

The acidogenic stage of AD is where TBBPA degradation takes place. However, due to the complexity of the media (domestic sewage), it remained unclear if the functional enzymes produced during the hydrolysis of complex macrocomponents would benefit TBBPA degradation. In addition, the microbiome profile during long-term operations under low concentrations of the pollutant had not yet been investigated. For this purpose, a strictly acidogenic and a hydrolytic-acidogenic reactor were operated and the results suggested that the microorganisms degrading TBBPA benefited from the electron flux taking place during glycolysis and further transformations

into short-chain fatty acids. It was also elucidated that the long-term exposure to TBBPA at environmentally relevant concentrations did not cause any major changes in the microbiome composition. Therefore, the third hypothesis was partially refuted, regarding hydrolysis, and confirmed, regarding the microbiome composition: Hydrolysis was a negligible step on the biodegradation of TBBPA and temporal sampling and sequencing of the biomass suggested that *Enterobacter* spp. and *Clostridium* spp. were the key players in the degradation process. The biomass from the continuous bioreactor was subjected to an enrichment with increasing concentrations of TBBPA up to 5 $mg \cdot L^{-1}$, which yielded results highlighting the effect of unrealistic concentrations frequently used in culture-dependent studies on the microbial community composition, as the *Clostridium* genus was outcompeted by the *Enterobacter* and *Klebsiella* genera.

Combining these findings, the stage of the anaerobic digestion and the putative TBBPA-degraders were elucidated. However, the results from the amplicon sequencing of the temporal profile of the biomass correlated to TBBPA removal efficiency did not confirm the identity of the active degraders. Additionally, by the genome sequencing of the TBBPA degrading organisms, the genes coding for proteins acting on the pollutant degradation could illustrate putative metabolic pathways. In this context, we used protein stable isotope-probing (protein-SIP) combined with metagenomics, which has become the cutting-edge technique in microbial ecology, for identifying the functional members in the microbial communities and metabolic pathways. The experiment presented in *Chapter 5* revealed that the bacterial populations actively degrading TBBPA were organisms affiliated to the *Phytobacter*, *Clostridium*, *Sporolactobacillus*, and *Klebsilla* genera. The incorporation of labelled carbons from the aromatic rings in the chemical structure of TBBPA corroborate and extend the previous results from multivariate statistical correlations on the identity of the organisms, as *Enterobacter* genus is affiliated to the same family as *Phytobacter* and *Klebsiella* genera, and the *Clostridium* genus was identified in both experiments (*Chapter 4* and *5*). Based on the predicted proteomes of these organisms, both cometabolic pathways and respiratory (dehalorespiration) mechanisms were possibly occurring in the bioreactor.

The combination of bioengineering parameters and state-of-art meta-omics approaches extended the knowledge on the anaerobic digestion of micropollutants, by combining results regarding the system efficiency, the role of the acidogenic stage, the microbiome profile, the identity of the organisms, and their predicted proteome. The relevance of fundamental research on

how a micropollutant is biologically converted is the first step to apply biochemical knowledge on industrial processes and we hope that this thesis has contributed to future researches in the environmental engineering and biotechnological fields.

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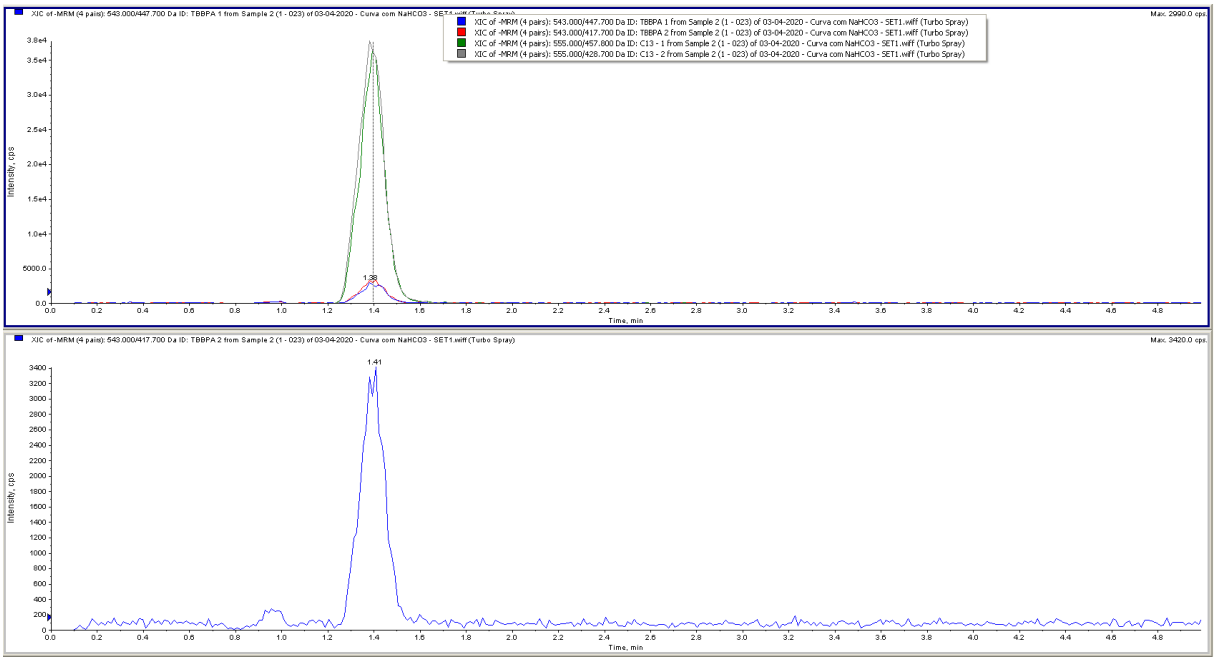
APPENDIX A

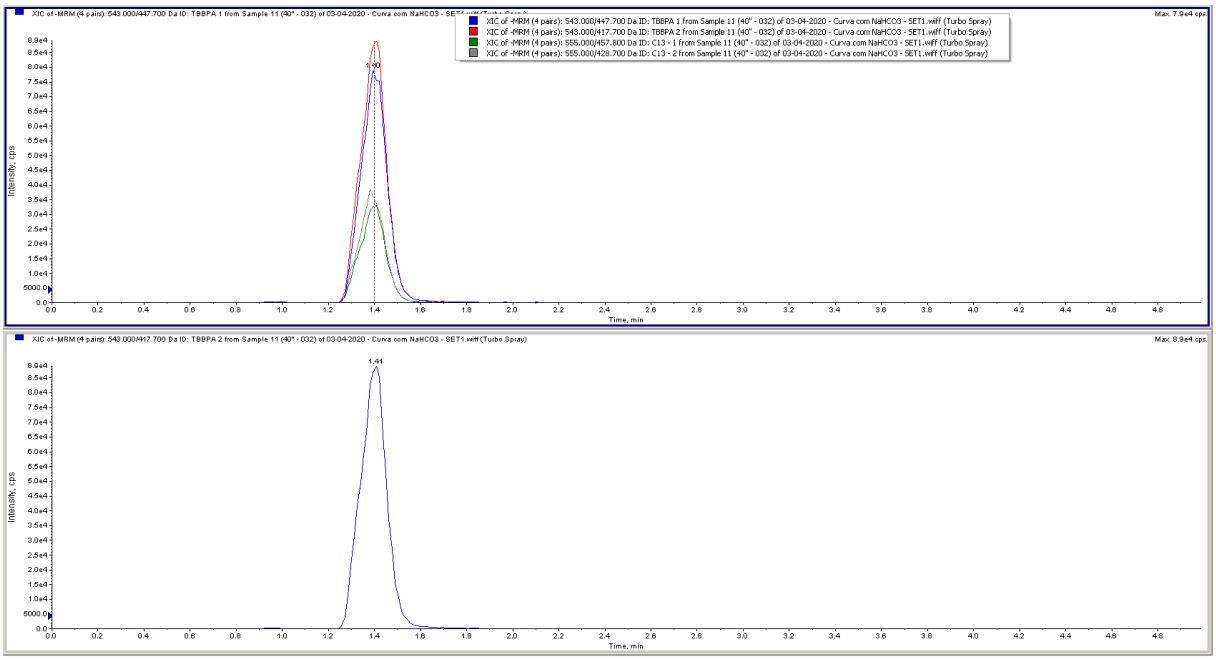
Application of dispersive liquid-liquid microextraction followed by HPLC/MS-MS analysis to determine Tetrabromobisphenol A in complex matrices

This supporting information contains 4 figures in which the mass chromatogram of TBBPA and $^{13}C_{12}$-TBBPA of the Limit of Quantification (LOQ) (Figures A.1 a, A.2 a, A.3 a, and A.4 a) and an intermediate concentration of the calibration curves (Fig. A.1 b, A.2 b, A.3 b, and A.4 b) for each studied matrix are presented.

Figure A.1 – Mass chromatogram of TBBPA and $^{13}C_{12}$-TBBPA at the LOQ (1 μg L^{-1}) (a) and intermediate concentration (40 μg L^{-1}) (b) for the lab-made domestic sewage matrix.

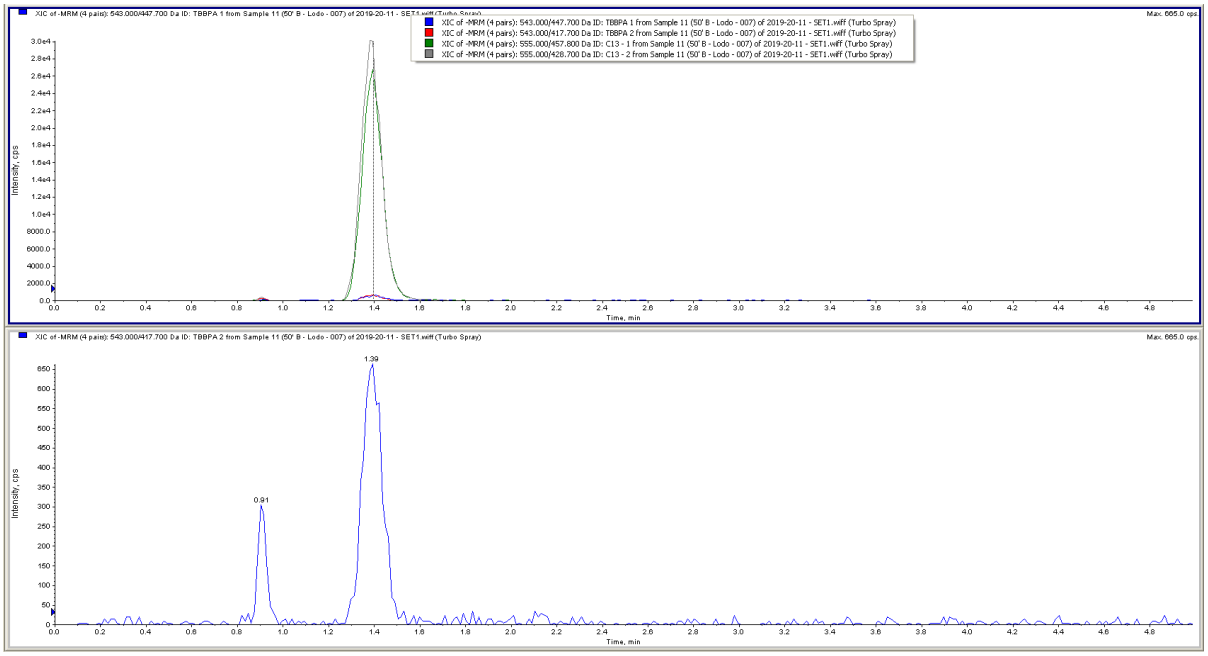


(a)

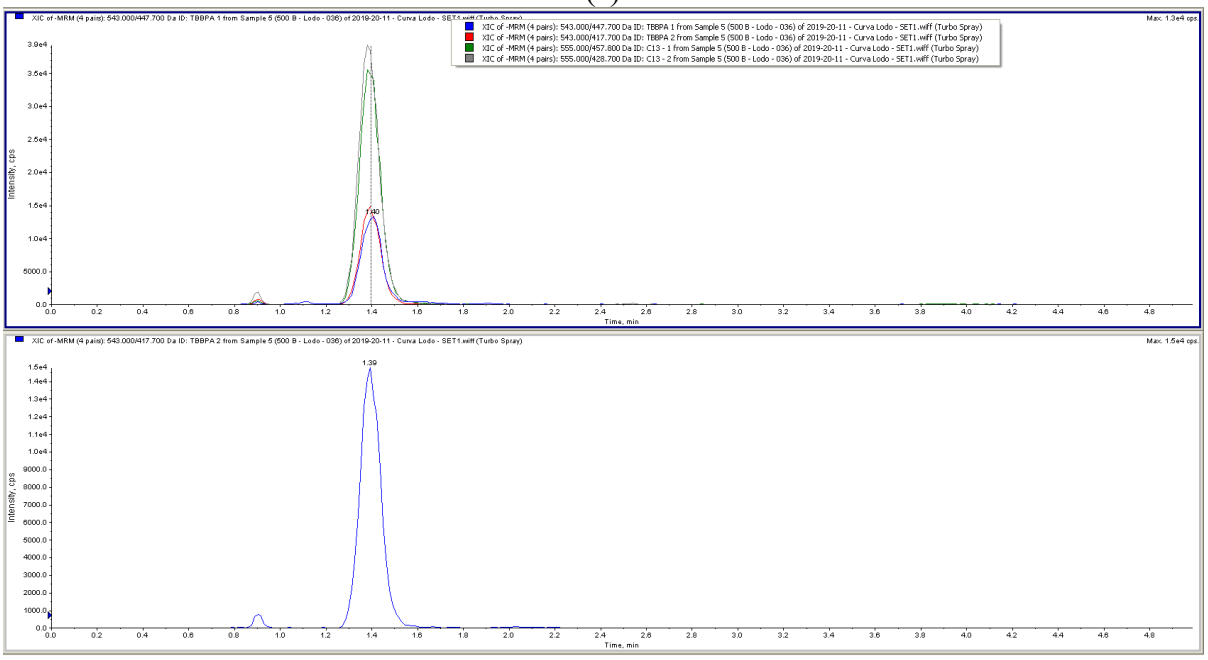

XIC of -MRM (4 pairs): 543.000/447.700 Da ID: TBBPA 1 from Sample 11 (40° - 032) of 03-04-2020 - Curva com NaHCO3 - SET1.wiff (Turbo Spray)
XIC of -MRM (4 pairs): 543.000/417.700 Da ID: TBBPA 2 from Sample 11 (40° - 032) of 03-04-2020 - Curva com NaHCO3 - SET1.wiff (Turbo Spray)
XIC of -MRM (4 pairs): 555.000/457.800 Da ID: C13 - 1 from Sample 11 (40° - 032) of 03-04-2020 - Curva com NaHCO3 - SET1.wiff (Turbo Spray)
XIC of -MRM (4 pairs): 555.000/428.700 Da ID: C13 - 2 from Sample 11 (40° - 032) of 03-04-2020 - Curva com NaHCO3 - SET1.wiff (Turbo Spray)
Max. 7.9e4 cps.
1.40
Intensity, cps
Time, min
XIC of -MRM (4 pairs): 543.000/417.700 Da ID: TBBPA 2 from Sample 11 (40° - 032) of 03-04-2020 - Curva com NaHCO3 - SET1.wiff (Turbo Spray)
Max. 8.9e4 cps.
1.41
Intensity, cps
Time, min

(b)

Figure A.2 – Mass chromatogram of TBBPA and $^{13}C_{12}$-TBBPA at the LOQ (50 ng g^{-1}) (a) and intermediate concentration (500 ng g^{-1}) (b) for the anaerobic sludge matrix.

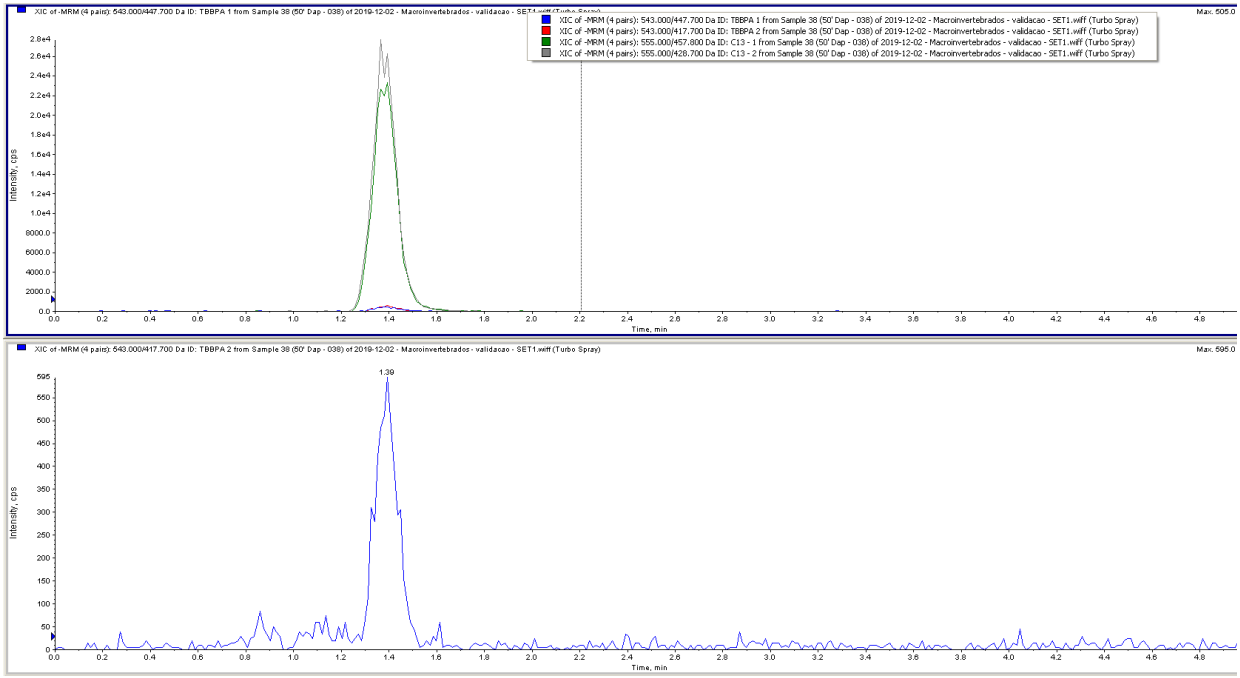


(a)

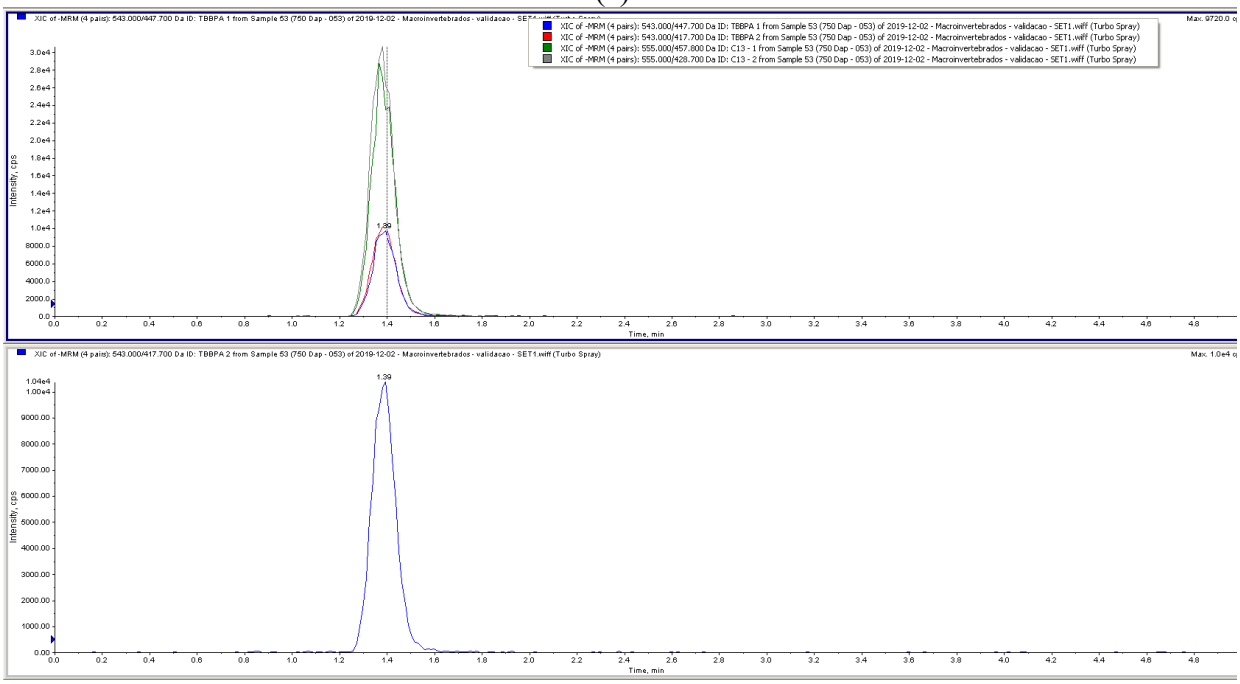


(b)

Figure A.3 – Mass chromatogram of TBBPA and $^{13}C_{12}$-TBBPA at the LOQ (5 ng g^{-1}) (a) and intermediate concentration (750 ng g^{-1}) (b) for the *Daphnia magna* matrix.

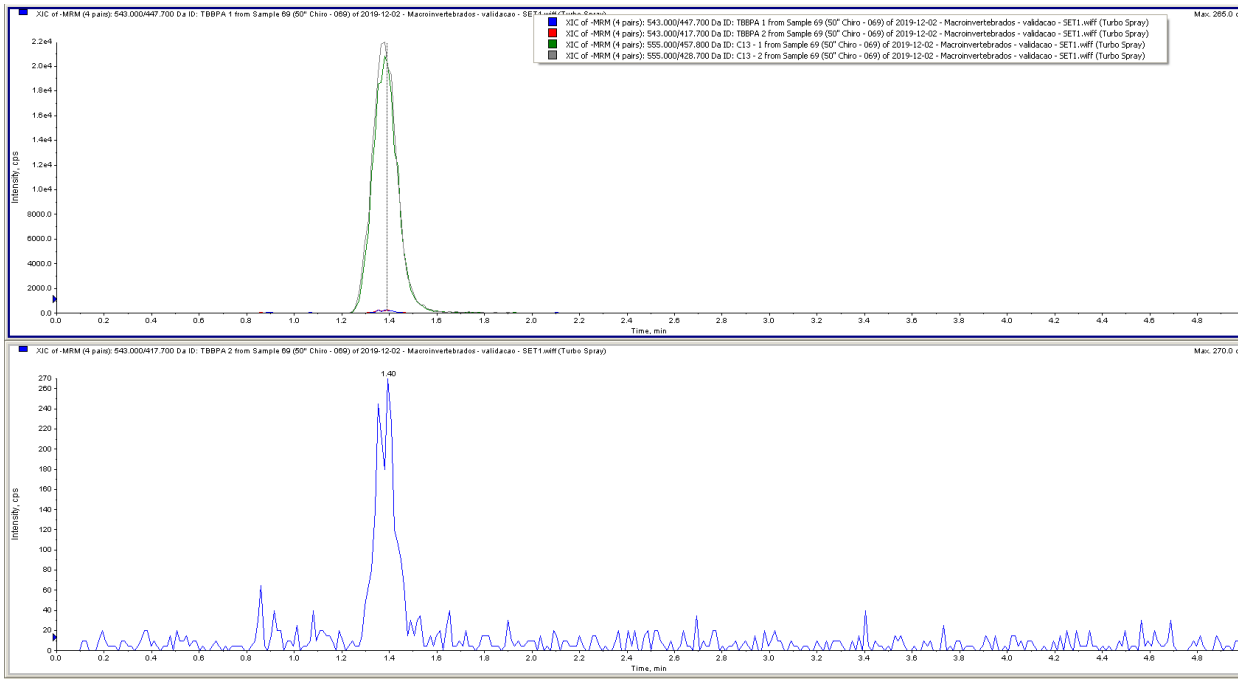


(a)

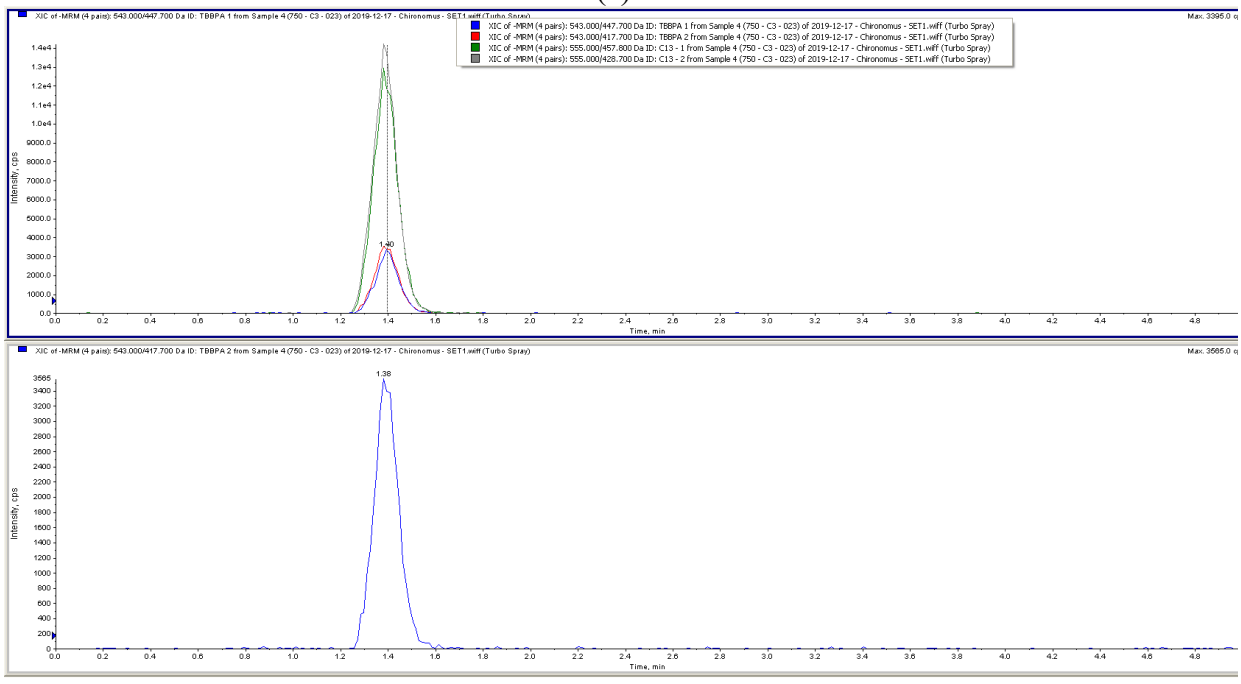


(b)

Figure A.4 – Mass chromatogram of TBBPA and $^{13}C_{12}$-TBBPA at the LOQ (50 ng g^{-1}) (a) and intermediate concentration (750 ng g^{-1}) (b) for the *Chironomus sancticaroli* matrix.



(a)



(b)

APPENDIX B

Tetrabromobisphenol A (TBBPA) anaerobic biodegradation occurs during acidogenesis

This supporting information contains figures, tables, and a description of the lab-made domestic sewage cited in the Chapter 3 of this thesis.

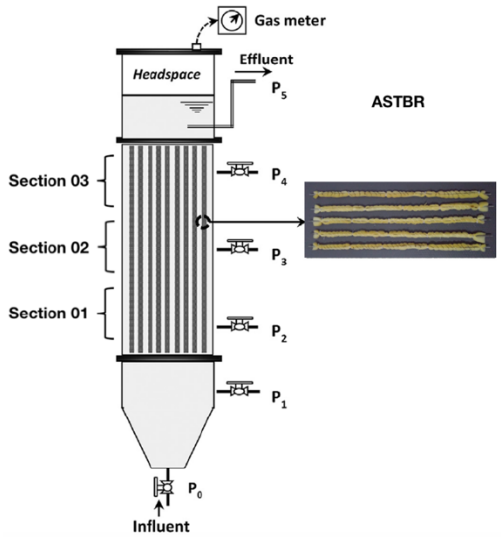


Figure B.1 – Schematic description of the anaerobic structured-bed reactors and withdrawing sample points. Adapted from Carneiro et al. (2019).

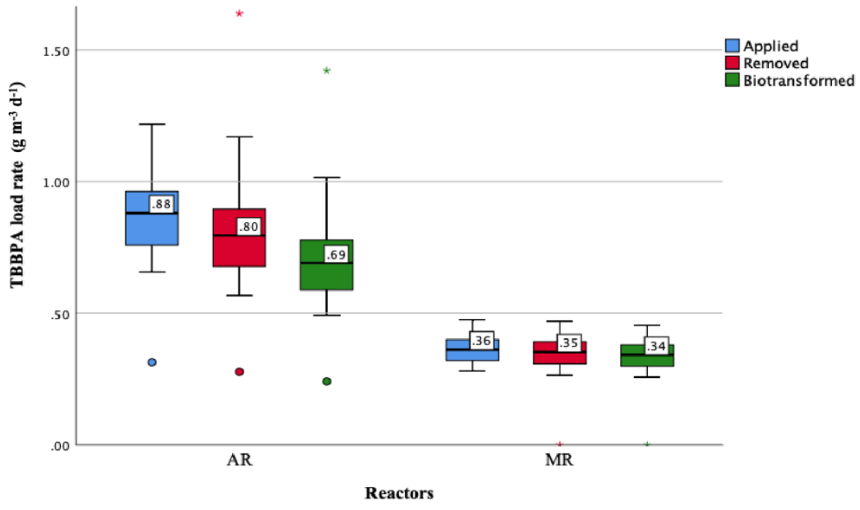


Figure B.2 –Applied, removed, and biotransformed TBBPA loading rates in the acidogenic (AR) and methanogenic (MR) reactors. The circles represent mild outliers, the stars represent extreme outliers.

Table B.1 – Biomass (adhered and suspended) profiles in the acidogenic (AR) and methanogenic (MR) reactors and TBBPA concentration at the end of the operational period.

Reactor's section	Adhered biomass (g VSS g $_{support\ material}^{-1}$)		Suspended biomass (g VSS L^{-1})		TBBPA concentration (µg g VSS^{-1})	
	AR	MR	AR	MR	AR	MR
#1 (0 to 18 cm)	1.47	0.58	1.95	6.58	33.8	4.7
#2 (18 to 36 cm)	0.59	0.52	1.75	0.09	18.7	4.1
#3 (36 to 52 cm)	0.70	0.47	1.55	0.08	28.8	4.1

Lab-made domestic sewage

The lab-made domestic sewage has the following composition (mg L^{-1}): beef extract (260), sucrose (45), soluble starch (142.5), NaCl (500), $MgCl_2.6H_2O$ (14), and $CaCl_2.2H_2O$ (9). A mineral solution was also added to the composition to avoid micronutrient limitation: nitriloacetic acid (NTA) (12.8), $FeCl_3.6H_2O$ (1.35), $MnCl_2.4H_2O$ (0.1), $CoCl_2.6H_2O$ (0.024), $CaCl_2.2H_2O$ (0.1), $ZnCl_2.4H_2O$ (0.1), $CuCl_2.2H_2O$ (0.025), H_3BO_3 (0.01), $Na_2MoO_4.2H_2O$ (0.024), $Na_2SeO_3.5H_2O$ (0.026), and $NiCl_2.6H_2O$ (0.12).

Additionally, in the RM feed composition, $NaHCO_3$ was added at a concentration of 500 mg L^{-1} to favor methanogenic conditions. The feed solution was prepared daily and maintained under refrigeration (4 °C).

APPENDIX C

TBBPA biodegradation in acidogenic systems: one step further on where and who

Wastewater composition

The strictly-acidogenic reactor (RAc) was fed with a readily fermentable carbon source (glucose, 937.5 $mg \cdot L^{-1}$) and the hydrolytic-acidogenic reactor (RHAc) was fed with a more complex carbon source (starch, 890 $mg \cdot L^{-1}$). The macronutrients added to the feed were ($mg \cdot L^{-1}$): NaCl (500), $MgCl_2.6H_2O$ (14), and $CaCl_2.2H_2O$ (9). A mineral solution was also added to the composition to avoid micronutrient limitation ($mg \cdot L^{-1}$): nitriloacetic acid (NTA) (12.8), $FeCl_3.6H_2O$ (1.35), $MnCl_2.4H_2O$ (0.1), $CoCl_2.6H_2O$ (0.024), $CaCl_2.2H_2O$ (0.1), $ZnCl_2.4H_2O$ (0.1), $CuCl_2.2H_2O$ (0.025), H_3BO_3 (0.01), $Na_2MoO_4.2H_2O$ (0.024), $Na_2SeO_3.5H_2O$ (0.026), and $NiCl_2.6H_2O$ (0.12). The feed solution was prepared daily and maintained under refrigeration (4 °C).

Table C. 1 – Descriptive statistics for Tetrabromobisphenol A (TBBPA), carbohydrates and Chemical Oxygen Removal (COD) removal.

Group Statistics					
	Reactor	N	Mean	Std. Deviation	Std. Error Mean
TBBPA removal (%)	RAc	28	99.3532	0.65851	0.12445
	RHAc	27	97.2659	1.09247	0.21025
Total Carbohydrates removal (%)	RAc	28	50.2679	6.68514	1.26337
	RHAc	27	30.2333	13.07081	2.51548
COD removal (%)	RAc	28	9.1071	3.92598	0.74194
	RHAc	27	5.5296	2.73479	0.52631

Table C.2 – Levene's Test for equality of variances and t-test for equality of means for TBBPA, carbohydrates and COD removal.

Independent Samples Test										
		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	T	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
TBBPA removal (%)	Equal variances assumed	2.791	0.101	8.618	53	0	2.08729	0.24221	1.60147	2.5731
	Equal variances not assumed			8.543	42.398	0	2.08729	0.24432	1.59438	2.5802
Carbohydrates removal (%)	Equal variances assumed	5.115	0.028	7.195	53	0	20.03452	2.78455	14.44942	25.61963
	Equal variances not assumed			7.117	38.417	0	20.03452	2.81492	14.33806	25.73099
COD removal (%)	Equal variances assumed	2.923	0.093	3.908	53	0	3.57751	0.91552	1.74122	5.41381
	Equal variances not assumed			3.933	48.307	0	3.57751	0.90966	1.74882	5.4062

Table C.3 – Descriptive statistics for organic acids production in the bioreactors.

Group Statistics					
	Reactor	N	Mean	Std. Deviation	Std. Error Mean
Formic acid	RAc	28	9.2104	4.76583	0.90066
	RHAc	28	7.2154	3.79712	0.71759
Acetic Acid	RAc	28	63.5679	29.80343	5.63232
	RHAc	28	59.6079	37.16031	7.02264
Butyric Acid	RAc	28	29.8564	34.17684	6.45882
	RHAc	28	31.9182	37.5516	7.09658
Lactic acid	RAc	28	53.8704	19.63335	3.71035
	RHAc	28	9.1221	8.41759	1.59077

Table C. 4 – Levene's Test for equality of variances and t-test for equality of means for organic acids production.

Independent Samples Test										
		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Formic acid	Equal variances assumed	2.156	0.148	1.732	54	0.089	1.995	1.15157	-0.31376	4.30376
	Equal variances not assumed			1.732	51.433	0.089	1.995	1.15157	-0.3164	4.3064
Acetic Acid	Equal variances assumed	1.746	0.192	0.44	54	0.662	3.96	9.00225	-14.08842	22.00842
	Equal variances not assumed			0.44	51.569	0.662	3.96	9.00225	-14.10792	22.02792
Butyric Acid	Equal variances assumed	0.06	0.807	-0.215	54	0.831	-2.06179	9.59572	-21.30004	17.17647
	Equal variances not assumed			-0.215	53.528	0.831	-2.06179	9.59572	-21.30393	17.18036
Lactic acid	Equal variances assumed	26.328	0	11.085	54	0	44.74821	4.03699	36.65454	52.84189
	Equal variances not assumed			11.085	36.602	0	44.74821	4.03699	36.56549	52.93094

APPENDIX D

Identification of TBBPA-degraders in anaerobic bioreactor at environmentally relevant concentrations by combined protein-based stable isotope probing and metagenomics

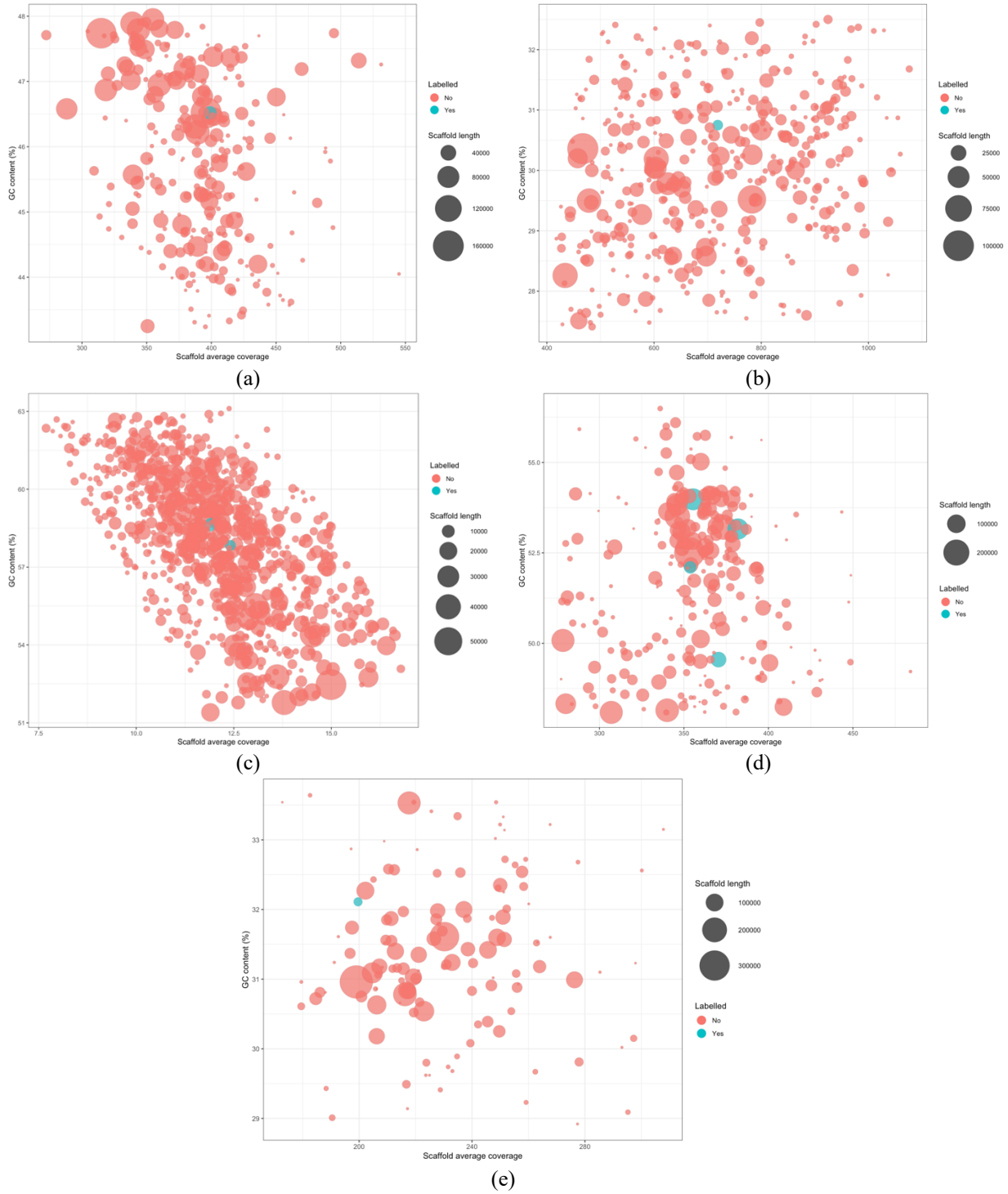


Figure D. 1 – Differential coverage binning of the extracted bins from the metagenome, shown as scaffold coverage and GC content. The dot sizes indicate the scaffold length. Colouring is according to the scalffold containing a template for the identified 13C-labelled proteins. Bin 1 (a), Bin 2 (b), Bin 3 (c), Bin 4 (d), and Bin 5 (e).