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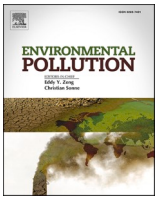

Proteogenomics identification of TBBPA degraders in anaerobic bioreactor☆

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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.

1. 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 μg 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 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., 2017).

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

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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·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., 2022). 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 μg·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; Mosbæk et al., 2016; 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 μg 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.

2. Material and methods

2.1. Microbial community and experimental setup

Batch reactors were inoculated with suspended biomass collected from a continuous acidogenic bioreactor treating synthetic wastewater contaminated with TBBPA (influent concentration of 50 μg L^{-1}). The main carbon source in the bioreactor was glucose at an organic load rate (OLR) of 12 kgCOD·m^{-3}·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 μg 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 L^{-1}), temperature at 30 °C, shaking at 200 rpm, and 5 gVSS·L^{-1}. The initial pH of the batch runs was 8.0 to enable complete TBBPA solubilization.

2.2. 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™ 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 analysed 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-10 A), and an Aminex HPX-87H column (300 mm, 7.8 mm, BioRad®) (Penteado et al., 2013).

2.3. 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.

2.4. 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 (Seemann, 2014). The functional annotation of the MAGs was performed through the MicroScope platform (Vallenet et al., 2020).

2.5. 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 unlabeled 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.

Table 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

3. Results

3.1. Degradation kinetics

The parameters of the adjusted kinetic model for the time-series profile of TBBPA are presented in Table 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.

3.2. 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. Fig. 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.

3.3. 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 five different bins on the metagenome. The peptides were identified as part of carbon-carbon lyases, transporters, oxidoreductases, and structural proteins. The labelled peptides, protein description, RIA value, phylogenetic affiliations of the peptide, and the samples in which they were found are presented in Table 2.

Labelled metagenome-assembled genomes (MAGs), predicted proteomes and the functional annotation from MicroScope®.

A total of 5 MAGs (Bin 1-5), containing coding sequences for the labelled peptides, were extracted using differential coverage binning of the assembly (Fig. 2). 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 3).

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

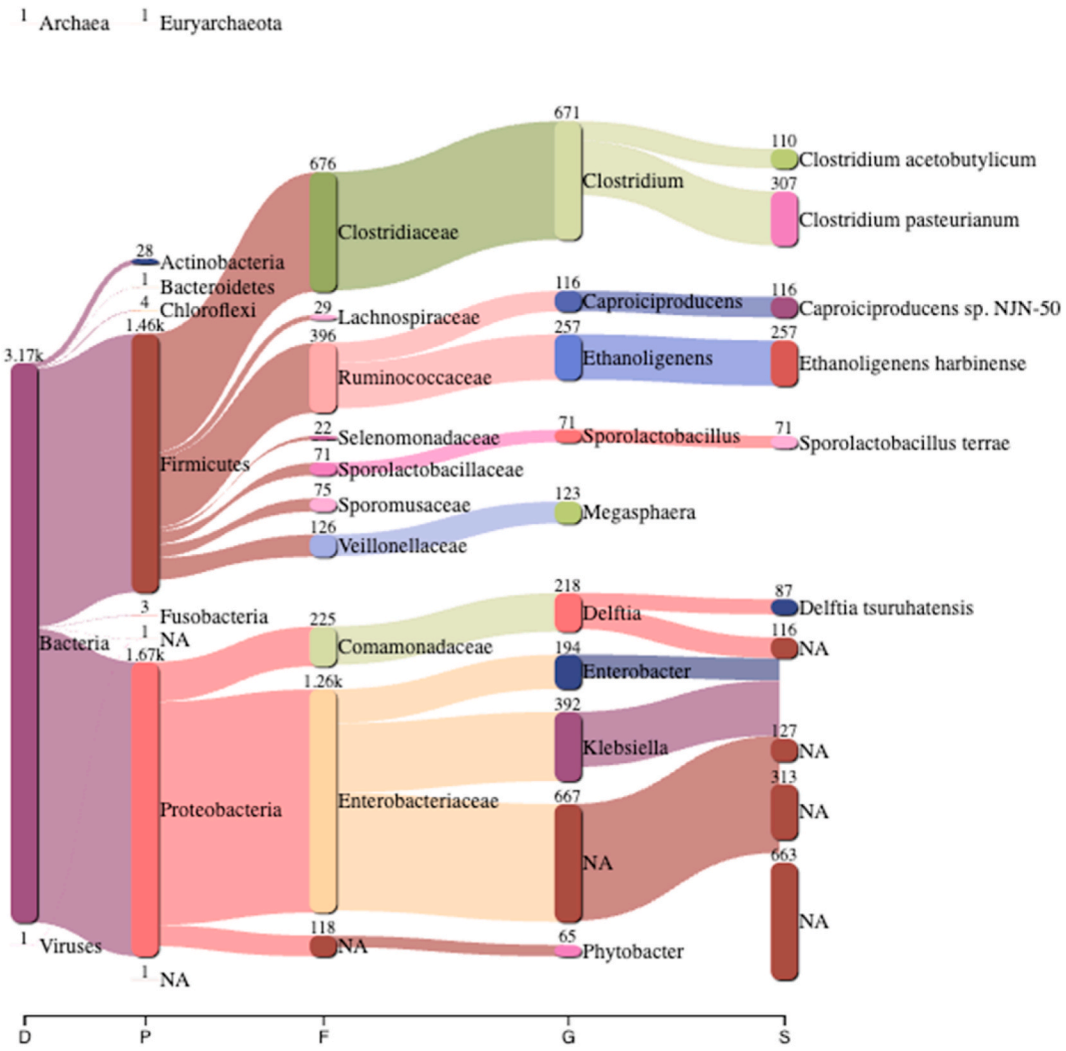

Fig. 1. - Sankey diagram displaying the composition of the microbiome from a acidogenic bioreactor and incubated with ^{13}C-TBBPA.

Table 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	RIA	Phylogenetic affiliation	
						Family	Species
1	50%	KPKJAHNK_38,736	SLVSEELTK	6-diphosphate aldolase	4.8	*Sporolactobacillaceae*	*Sporolactobacillus terrae*
2		KPKJAHNK_57,630	LTSSPAAVSWGNNR	Hypothetical protein	3.7	*Clostridiaceae*	*Clostridium pasteurianum*
3		WP_028114164.1	LASTWEGIQAAR	Transaldolase	4.1	*Enterobacterceae*	*Klebsiella pseudopneumoniae*
4	75%	KPKJAHNK_28,756	DAEQDIILQEMYDK	LPS assembly lipoprotein LptE	0	*Enterobacterceae*	*Phytobacter ursingii*
5	99.9%	KPKJAHNK_05461	AAFDQWVAK	Cytochrome bo3 quinol oxidase subunit 2	4.7	*Enterobacterceae*	*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	*Enterobacterceae*	*Phytobacter ursingii*
8		KPKJAHNK_18,028	EFVESLETPR	6-Phosphogluconate Dehydrogenase	4.3	*Enterobacterceae*	*Phytobacter ursingii*
9		KPKJAHNK_42,614	PVINHNLSAINANR	Flagellin	3	*Clostridiaceae*	*Clostridium pasteurianum*
10		WP_042394572.1	QETGDNVTIR	Sulfate ABC transporter Substrate-binding protein	5	*Enterobacterceae*	*Klebsiella pseudopneumoniae*

Proteobacteria (bins 3 and 4). These bins included representatives of *Sporolactobacillus terrae* (bin 1), *Clostridium pasteurianum* (bin 2), *Klebsiella 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 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 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

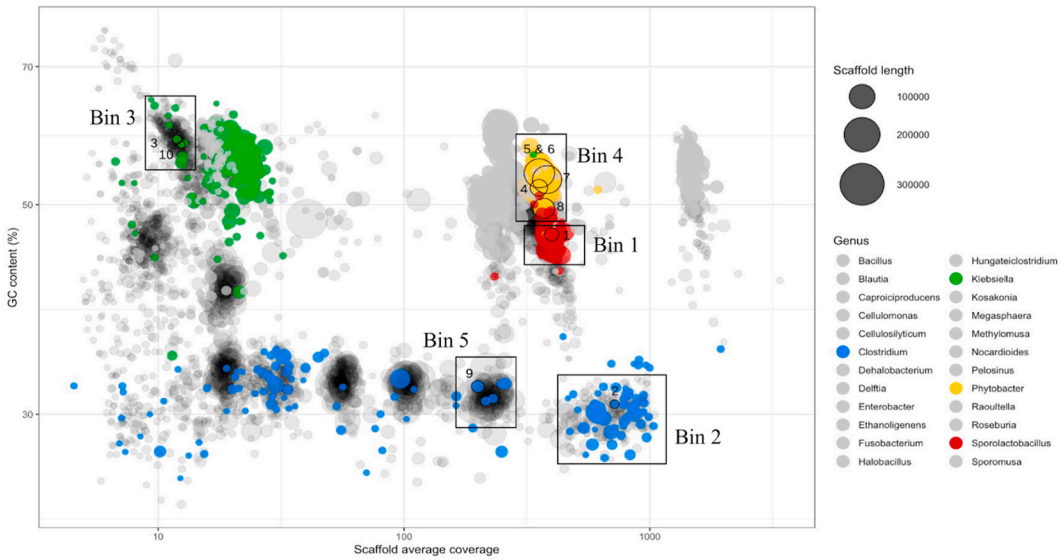

Fig. 2. Differential coverage binning of the combined metagenome, shown as scaffold coverage and GC content. The dot sizes indicate the scaffold length, and with a minimum scaffold length of 6000 bp. a) Colouring is according to taxonomy (genus level), and only the genera containing a template for the 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 bins 1 to 5.

Table 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

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).

4. Discussion

The aim of the study was to identify and elucidate microorganisms actively involved in the cometabolic degradation of the micropollutant TBBPA at concentrations close to what has been detected in the environment.

4.1. 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., 2021).

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 μg 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 (≥20%) and labeling ratio (LR) (≥10%) values (Taubert et al., 2012; Morris et al., 2012; Vogt et al., 2016; Mosbæk et al., 2016; de Jonge et al., 2021). However, this is the first study in which the labelled compound was used in the μg·L^{-1} as part of a cometabolism together with glucose as the main energy and carbon source. Unrealistically high concentrations of micropollutants (mg·L^{-1}), compared to those occurring in natural systems, can induce specific gene

Table 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 (1968)
		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. (2003)
		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 *b*556 (Fdn) subunit	Formate dehydrogenase allows the use of formate as major electron donor during anaerobic respiration	Jormakka et al. (2002)
		Probable efflux pump		
		Aromatic amino acid exporter YddG		Blattner et al. (1997)
		Cytochrome bd-II ubiquinol oxidase subunit 2	Energy metabolism; Electron transfer	Dassa et al. (1991)
		Cytochrome *b*561	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)

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.

4.2. Phylogenetic characterization of the identified TBBPA degraders

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 Júnior 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., 2021), 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.

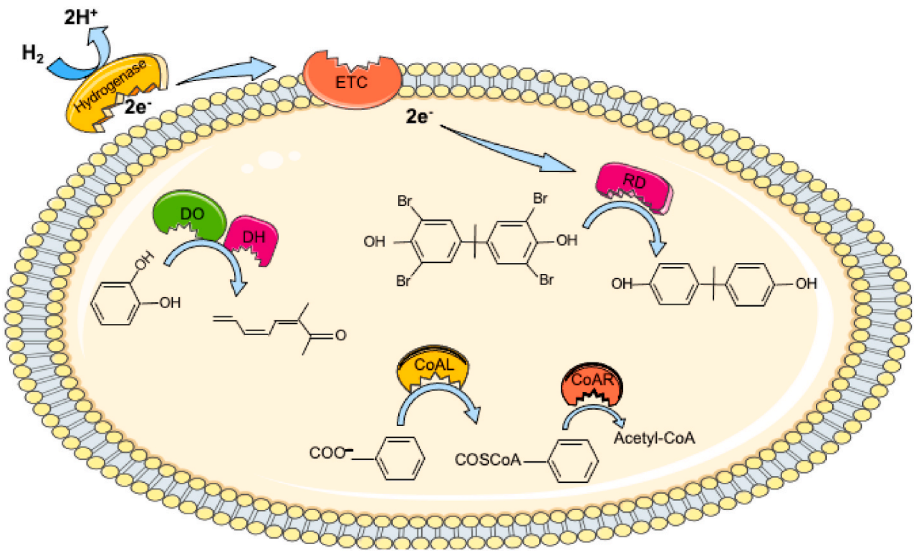

Fig. 3. – Encoded enzymes from *Sporolactobacillus* and *Phytobacter* metagenome-assembled genomes possibly involved in TBBPA degradation. ETC: electron transfer carrier; RD: reductive dehalogenase; DO: dioxygenase; DH: dehydrogenase; CoAL: CoA ligase; CoAR: CoA reductase.

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 Júnior 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.

4.3. Predicted proteomes from metagenome-assembled genomes and functional annotation

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 2 and 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; Häggblom, 1992; Fetzner, 1998). Monoxygenases and dioxygenases are essential enzymes for the hydroxylation and cleavage of aromatic ring structures (Häggblom, 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 2 and 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.

According to the predicted proteomes and the functional annotation of the genomes of the active TBBPA degraders, the most likely degradation pathways would involve 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 utilized by both *Sporolactobacillus* and *Phytobacter* organisms are illustrated in Fig. 3.

5. 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.

Authors statement

W.V. Macêdo: Conceptualization, Methodology, Data curation, Formal analysis, Investigation, Writing – original draft, review, and editing; **J. S. Poulsen**: Methodology, Data curation; Formal analysis, Investigation, Writing – original draft, review, and editing; **M. Zaiat**: Conceptualization, Methodology, Resources, writing, review, editing, Supervision, Project administration, and Funding acquisition; **J. L. Nielsen**: Conceptualization, Methodology, Resources, writing, review, editing, Supervision, Project administration, and Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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