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Degradation of Brominated Organic Compounds (Flame Retardants) by a Four-Strain Consortium Isolated from Contaminated Groundwater

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Abstract: Biodegradation of pollutants in the environment is directly affected by microbial communities and pollutant mixture at the site. Lab experiments using bacterial consortia and substrate mixtures are required to increase our understanding of these processes in the environment. One of the deficiencies of working with environmental cultures is the inability to culture and identify the active strains while knowing they are representative of the original environment. In the present study, we tested the aerobic microbial degradation of two brominated flame retardants, tribromo-neopentyl alcohol (TBNPA) and dibromo neopentyl glycol (DBNPG), by an assembled bacterial consortium of four strains. The four strains were isolated and plate-cultured from a consortium enriched from the impacted groundwater underlying the Neot Hovav industrial area (Negev, Israel), in which TBNPA and DBNPG are abundant pollutants. Total degradation (3–7 days) occurred only when the four-strain consortium was incubated together (25 °C; pH −7.2) with an additional carbon source, as both compounds were not utilized as such. Bacterial growth was found to be the limiting factor. A dual carbon–bromine isotope analysis was used to corroborate the claim that the isolated strains were responsible for the degradation in the original enriched consortium, thus ensuring that the isolated four-strain microbial consortium is representative of the actual environmental enrichment.

Keywords: substrate mixtures; assembled microbial consortium; isotopic fractionation; syntrophy; simultaneous utilization; brominated flame retardants

1. Introduction

Halogenated organic compounds are one of the largest groups of chemicals that cause environmental contamination [1]. Their high resistance to degradation often results in their accumulation in the environment, with microbial degradation being the primary process determining their fate [2]. The two brominated flame retardants (BFR), i.e., dibromoneopentyl glycol (DBNPG) and tribromoneopentyl alcohol (TBNPA), are from the abundant pollutants found in the groundwater underlying the Neot Hovav industrial zone, Israel [3]. Numerous organic compounds pollute this aquitard and sustain a rich microbial community, which is spatially changing due to contamination [4]. BFRs are known for their widespread adverse health effects, with TBNPA being defined as a moderate aquatic and human hazard by the EPA [5] and DBNPG defined carcinogenic in rodents [6].

At sites polluted by multiple contaminants, microbial consortia rather than single strains have proven to be more efficient in biodegradation and bioremediation processes [7], as often observed for biodegradation petroleum and polycyclic aromatic hydrocarbons [8,9]. This stems from the high metabolic diversity and resilience of microbial consortia in relation to single strains [1,10,11]. Halo-respiring bacteria have exhibited a similar tendency. For example, several *Dehalobacter* spp. have been reported to grow only in co-cultures or consortia, leading to questions on the role of syntrophic interactions in dehalogenation processes [12]. Syntrophic relations, or the use of one microbe's byproduct by another, are microbially advantageable in nutrient-poor environments, especially for microbial adaptation and biodegradation of complex compounds. Nevertheless, there is still limited knowledge of these relationships [13–16].

Although more frequently exhibited and demonstrated in anoxic conditions, syntrophic relations also occur in aerobic environments [13,14,16]. Ronen et al. (2005) showed that communalistic interactions with other soil microorganisms were necessary for the *Achromobacter piechaudii* strain TBPZ to biodegrade 2,4,6-tribromophenol in a contaminated desert soil due to the strain's autotrophy to amino acids and vitamins [16]. Morris et al. (2013) observed that in co-enrichments of methanotrophs and methylotrophs, growth is enabled by the oxidation of methanol by the methylotrophs [14]. Tao et al. (2017) demonstrated that the co-culture of *Bacillus subtilis* ZF3-1 with an indigenous bacterial consortium predominated by the *Burkholderiales* order displayed an enhanced ability for crude oil degradation in aerobic conditions, which is likely to occur through the removal of *n*-alkane [17]. These studies indicate that mixtures of strains and/or contaminants enhance biodegradation.

Mixtures of substrates affect biodegradation readability, resulting in degradation stimulation or inhibition of a single substrate in the mixture. Elucidating these substrate interactions is essential, as most polluted sites include a broad spectrum of compounds. Mutual inhibitory and enhancement effects induced by substrate mixtures in aerobic conditions have been demonstrated in the literature [18–24].

The biodegradation of halogenated organic compounds includes a variety of enzymatic mechanisms [25]. An essential route of halogenated organic degradation is dehalogenation, i.e., halogen–carbon bond cleavage, which can proceed either enzymatically or by spontaneous chemical dehalogenation of unstable intermediates [26]. Enzymatic dehalogenation involves multiple mechanisms (hydrolysis, reduction, or oxygen-dependent mechanisms) by which halogen atoms are removed. Hydrolysis includes halogen replacement through nucleophilic substitution by water derived from a hydroxyl group; reduction involves halogen replacement by hydrogen; and oxygenolytic dehalogenation, which is catalyzed by monooxygenase or dioxygenase, results in the incorporation of one or two atoms of molecular oxygen into the substrate [2,26–28].

In the past decade, a multi-elemental isotope approach has been developed to understand complex degradation pathways, both biotic and abiotic. Dual compound specific isotope analysis (CSIA) may help us to understand degradation mechanisms, assist in differentiating between mechanisms, and clarify contaminant fate in the environment [29–34]. Usually significant isotope fractionation occursduring bond cleavage and its magnitude depends on the degradation mechanism [32,35]. However, isotopic analysis of bromine, a less commonly used technique in CSIA, has not been extensively investigated [29]. An application of a $^{13}C/^{12}C$-$^{81}Br/^{79}Br$ isotope analysis to study the biotic transformation pathway of tribromoneopentyl alcohol by groundwater enrichment culture was demonstrated previously [36]. The enriched groundwater consortium was microbially characterized, but the strains involved in the process itself remained unidentified.

The objectives the current work are to (1) isolate and culture the specific strains that are essential for the degradation of dibromoneopentyl glycol (DBNPG) and tribromoneopentyl alcohol (TBNPA), (2) examine the isolates' preference in degrading one compound over the other, and (3) use the isotopic tools (carbon and bromine fractionation) to elucidate whether the isolates are characteristic for the microbial activity in the original enrichment consor-

tium. To this end, pure strains were isolated from a previously reported consortium [28], incubated in different combinations of TBNPA and DBNPG amendments, along with the use of auxiliary amendments (glucose, yeast extract, and vitamin mix) to determine the influence of supplements on degradation activity. Additionally, the $^{13}C/^{12}C$-$^{81}Br/^{79}Br$ isotope analysis was carried out to compare the degradation mechanism of both compounds and compare the degradation mechanism by the co-culture of the isolated strains to the original consortium.

2. Materials and Methods

2.1. Chemicals and Solvents

TBNPA (>98% pure) was obtained from TCI chemicals (Japan), and ICL Israel supplied DBNPG. Methanol, dichloromethane, di-propanol, and acetonitrile were all high-performance liquid chromatography (HPLC) grades (supra-gradient, Bio-Lab, Jerusalem, Israel).

2.2. Isolates

Consortia enriched under aerobic conditions from Neot Hovav groundwater were formerly established and used in this study for isolating TBNPA and DBNPG degrading strains. In short, enrichment included a groundwater inoculum (10 mL) mixed with 90 mL growth medium and either TBNPA or DBNPG at an initial concentration of 100 mg/L [28]. Consortia were streaked on R2A agar plates (BD Difco, Sparks, MD, USA) without DBNPG and TBNPA, and distinctive colonies were further purified on the R2A medium. Strains were identified by 16S rRNA gene sequencing (HyLab, Rehovot Israel). A total of four and five isolates were obtained from the DBNPG and TBNPA original groundwater consortia, respectively.

To assess whether the isolates can degrade TBNPA and DBNPG, each strain was grown in a liquid growth medium by amendment of yeast extract (100 mg L^{-1})/glucose (100 mg L^{-1}) and TBNPA/DBNPG (100 mg L^{-1}). Individual strains did not exhibit TBNPA/DBNPG degradation, leading to the investigation of different strain combinations. Finally, a four-strain consortia, which successfully degraded TBNPA and DBNPG, was selected for further study. More detail is available in the Supplemental Materials on how strains were screened for degradation. Two techniques measured individual strain biomass of the four-strain consortium's (before combining the strains to the consortium) colony-forming units (CFU/mL) with a series of six serial dilutions as described in [37] and by optical density measurements (OD600) (BioMate 5, Thermo Spectronic, Thermo Fisher Scientific, Waltham, MA USA). The correlation between the two was calculated through the dual measurements of both. The calibration of CFU to OD yields the following relationships: CFU $mL^{-1} = 10^8 \times$ OD600. In all experiments, an inoculum (with growth medium ratio of 1:10) was kept throughout all experiments.

2.3. Degradation

TBNPA and DBNPG degradation by the four-strain consortium was tested with three different amendments: yeast extract (BD Difco, Sparks, MD, USA; 100 mg L^{-1}), DL-vitamin mix (2 mL L^{-1}, composition in Supplementary Materials), and glucose (Sigma Aldrich, Tokyo, Japan, 100 mg L^{-1}). All experiments were implemented under aerobic conditions, and incubated in the dark at a constant temp of 25 °C at pH 7.2 on an orbital shaker at 120 rpm. Changes in the substrate TBNPA or DBNPG concentration was monitored during incubation using high-performance liquid chromatography (HPLC; Agilent 1100 series, Palo Alto, CA, USA); OD600 measured biomass growth, and bromide was quantified as previously described [36]. Aseptic technique was applied through all experiments and sampling events.

2.3.1. TBNPA and DBNPG Degradation Kinetics with Yeast Extract

Degradation kinetic experiments were carried out in a 450 mL growth medium (see Supplementary Materials) inoculated with 50 mL of the active four-strain consortium at

the stationary phase (OD600 ≈ 0.07), with an initial TBNPA or DBNPG concentration of 100 mg L^{-1}. Experiments were conducted in duplicates and accompanied by an abiotic control. During incubation, 2 mL samples were analyzed for TBNPA or DBNPG concentrations until the complete disappearance of TBNPA or DBNPG.

2.3.2. Simultaneous Biodegradation of TBNPA and DBNPG with Yeast Extract

Simultaneous biodegradation of TBNPA and DBNPG by the four-strain consortium was tested to examine whether the community exhibits a preference for one compound over the other. The four-strain consortium was acclimated to DBNPG and TBNPA and was inoculated in experimental flasks after being transferred three times through DBNPG and TBNPA amended media. Experiments were conducted in triplicate, with TBNPA and DBNPG (50 mg L^{-1} each) amended to the medium (90 mL of growth medium inoculated with 10 mL of active four-strain consortium in the stationary phase). Two biotic controls consisted of the four-strain consortia with either DBNPG or TBNPA at 100 mg L^{-1}, and a negative abiotic control was amended with DBNPG and TBNPA 100 mg L^{-1} each. The samples (i.e., DBNPG, TBNPA, and bromide concentrations) were analyzed for optical density (OD600).

2.3.3. Individual and Simultaneous Degradation of TBNPA and DBNPG with Vitamin Mix or Glucose Amendment

Experiments were carried out separately with 45 mL of growth medium inoculated with 5 mL of the active four-strain consortium in the stationary phase. When amended together, TBNPA and DBNPG initial concentrations were 50 mg L^{-1} each, whereas when amended separately, initial concentrations were 100 mg L^{-1}. Experiments were conducted in duplicates and accompanied by an abiotic control.

2.4. Isotopic Fractionation

Experiments were conducted at an initial volume of 0.5 L and inoculated with the active four-strain consortium (450 mL growth medium inoculated by 45 mL active consortium). Flasks were sampled throughout incubation daily for TBNPA or DBNPG concentrations. Sampling frequency and volume increased when the remaining substrate fraction (f) was below 15% of the initial concentration to capture the isotopic effect. Experiments were carried out in triplicate and accompanied by an abiotic control. For isotope analysis, TBNPA or DBNPG were extracted from the medium by a solid-phase extraction (SPE) method [36]. The filtered four-strain consortium samples were passed through SPE cartridges (Strata C18-E, Phenomenex, Torrance, CA, USA), previously conditioned with dichloromethane, 2 mL methanol, and 2 mL of double-distilled water. Cartridges were dried for 20 min under vacuum and eluted by 2 mL methanol [3]. The extract was further concentrated when necessary, under a gentle flow of nitrogen to reach a 200 mg L^{-1} concentration of TBNPA or DBNPG.

2.5. Analytical Methods

TBNPA and DBNPG concentrations were determined by high-performance liquid chromatography (HPLC; Agilent 1100 series, Palo Alto, CA, USA) using a Supelcosil™ LC-18 column (Supelco, Bellefonte, PA, USA), with an eluent of 45:55 acetonitrile to water ratio at a flow rate of 1.5 mL min^{-1}. The concentrations of both compounds were quantified at 210 nm using a calibration curve. The detection limit of 1 mg L^{-1} was determined along with an analytical error of ±2%. Carbon and bromine isotope analyses were performed according to the methods described earlier [38]. Carbon isotope ratios were expressed relative to an international standard (V-PDB) in ‰ units. $^{81}Br/^{79}Br$ ratios were obtained from the coupling of a gas chromatograph (Hewlett-Packard 5890) to a multicollector-inductively coupled plasma mass spectrometer (MC-ICPMS Nu Instruments, Wrexham, UK) analysis after correction by a Sr external spike as previously reported [39]. Bromine isotope ratios during the experiment (specific time points—R_{sample}) in per mil units were

expressed relative to the bromine isotope ratio of the sample at time zero according to Equation (1):

$$\delta^{81}Br,\ ‰ = \left[\frac{R_{sample} - R_{t0}}{R_{sample}}\right] \times 1000 \quad (1)$$

where R_t is the isotopic ratio in the sample at a given time in the experiment and R_{t0} is the ratio at time zero. The samples were analyzed three times each for carbon and bromine isotope compositions.

2.6. Calculations

The isotopic enrichment factor was derived using the Rayleigh equation, which correlates the shift in the isotopic composition and extent of degradation (Equation (2)):

$$\ln\left(\frac{R_{x,t}}{R_{x,0}}\right) = \varepsilon \cdot \ln\ f \quad (2)$$

where ε is the bulk isotopic enrichment factor observed for the compound, f is the extent of degradation (often described as C/C_0, where C_0 and C are the compound concentrations at times zero and t, respectively). R_x is the isotope ratio of the studied element (carbon or bromine) in the substrate at times zero and t. The carbon and bromine isotope enrichment factor, ε, was obtained as a slope of the linear regression of the experimental points plotted as the natural logarithm of the isotopic enrichment, $R_{x,t}/R_{x,0}$, against the natural logarithm of the degradation extent, f.

3. Results and Discussion

3.1. Microbial Isolates

In our former work [36], we established TBNPA and DBNPG enrichment cultures in which total of nine strains were isolated in the current study (five and four isolates for each consortium, respectively; Table 1). Two strains of *Pseudomonas citronellolis* were found in both groups of isolates. When amended with a carbon source (yeast extract/100 mg L^{-1} glucose) and TBNPA or DBNPG, all nine individual strains exhibited growth but no TBNPA or DBNPG degradation.

Table 1. Strain ID used in experiments with identified genus and species with initial density given for the four-strain consortium, which was seen able to degrade both TBNPA and DBNPG.

ID	Strain (Highest Similarity, 99%)	Gene Bank Accession Number	OD 600 (Initial Density CFU/mL)
DB2	*Pseudomonas citronellolis*	KY229738	0.001 (1.10×10^5)
DB3	*Gordonia sihwensis*	KY229739	0.001 (1.70×10^5)
DB4	*Shinella zoogloeoides*	KY229740	0.004 (4.90×10^5)
DB5	*Microbacterium oxydans*	KY229741	0.027 (3.00×10^6)
TB1	*Pseudomonas aeruginosa*	KY229734	
TB2	*Delftia tsuruhatensis*	KY229735	
TB3	*Pseudomonas citronellolis*	KY229736	
TB4	*Sphingobacterium siyangense*	KY229752	
TB5	*Microbacterium paraoxydans*	KY229753	

Different strain combinations originating either in the TBNPA or DBNPG enrichment culture were tested for degradation. DBNPG and TBNPA degradation occurred only with a combination of the four isolates from the DBNPG enrichment culture, strains DB2–DB5, and an additional carbon source (screening and degradation experiments to determine this strain combination are described in the Supplementary Materials). Therefore, these

four strains were used for all subsequent experiments. In the formerly established DBNPG enrichment culture, from which the strains were isolated [36], DB2–DB5 were observed in varying abundances (data derived from Illumina MiSeq sequencing). *Gordonia sihwensis* (DB3) and *Microbacterium oxydans* (DB5), belonging to the *Actinomycetales* order, appeared at a relatively low abundance at <2% in both TBNPA and DBNPG enrichments. *Pseudomonas* (DB2), on the other hand, is relatively abundant in the TBNPA enrichment (29.3%), while in the DBNPG enrichment, it was only 7.4%. *Shinella zoogloeoides* (DB4), belonging to the *Betaproteobacteria* class, showed an opposite trend, i.e., high in the DBNPG enrichment (21%) and low in the TBNPA enrichment (3%).

These isolates can most likely catalyze an oxidative mechanism involving a mono or dioxygenase, as was previously demonstrated [36]. In addition, several monooxygenases have been identified previously in strains similar to each of the four isolates, pointing to their potential in TBNPA and DBNPG oxidation [40–46]. For example, *Pseudomonas citronellolis* UAM-Ps1 co-metabolically transforms methyl tert-butyl ether (MTBE) to tert-butyl alcohol with n-pentane or n-octane as a gratuitous inducer of alkane hydroxylase (AlkB) [47]. In our study, it was unclear which of the strains was directly involved in the degradation of the compounds. The necessity to incubate all four strains to facilitate the degradation of DBNPG and TBNPA implies that some form of syntrophic relationship exists between the four strains. Syntrophic relationships between bacteria may be in the form of (1) metabolic complementation, in which the degrading bacteria depend on auxiliary strain/s that provide nutrients and growth factors or remove toxic metabolites, or (2) associated metabolism, in which all consortium members utilize metabolites through cross feeding [48]. Since all four strains are necessary for initiating the degradation of the target compounds, we suggest that the relationship between them is a form of metabolic complementation.

3.2. DBNPG and TBNPA Degradation

DBNPG and TBNPA (Figure 1) degradation by the four-strain consortium (*Pseudomonas citronellolis*, *Gordonia sihwensis Shinella zoogloeoides*, *Microbacterium oxydans*) was tested with and without the amendments yeast extract (YE), vitamin mix, or glucose. As degradation did not occur without any additions, the amendments were chosen to unravel the limiting factor for degradation and to examine whether metabolic complementation between the four strains exists. Degradation kinetics were tested with separate and mixed substrates: yeast extract (YE, 100 mg L^{-1}), DL-vitamin mix (2 mL/l), and glucose (100 mg L^{-1}) (Table 2).

Degradation was observed with all three amendments. Nevertheless, while yeast extract and glucose promoted substantial bacterial growth in the vitamin mix, amended media growth was minimal. Amendment type also affected degradation rates. Degradation was rapid with glucose and YE, with total degradation completed within 3–7 days. DL-vitamin mix amendment led to significantly slower degradation rates, with around 1–2 months necessary for complete degradation (Table 2). A clear positive correlation was observed between microbial growth and degradation rates. The ability of the strains to degrade the compounds regardless of the differences in amendments indicates that the medium deficiency is the carbon source and that the four-strain consortium does not utilize TBNPA and DBNPG as a carbon source for growth, e.g., carbon is the limiting factor for degradation. All the additional components that were required, such as different nutrients, vitamins, and more, were provided through metabolic complementation. It was suggested that the consortium utilizes the impurities in the DL-vitamin mix and the vitamins themselves as a carbon source (the DL-vitamin mix does not contain carbon). The low concentrations and complex nature of the vitamin molecules caused slower biomass growth and relatively lower degradation rates. Controls with no amendment did not lead to degradation.

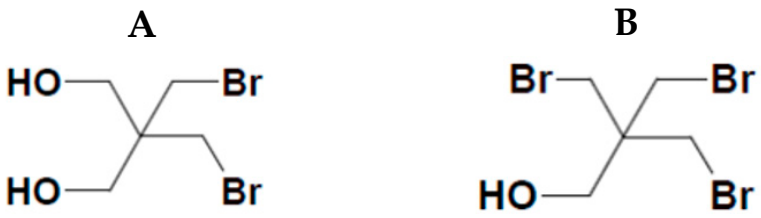

Figure 1. Molecular structure of (**A**) dibromoneopentyl glycol (DBNPG); (**B**) tribromoneopentyl alcohol (TBNPA).

Table 2. Consortium biomass (OD600) at the end of the exponential phase, and time for complete degradation of TBNPA, DBNPG, or both TBNPA and DBNPG in consortia amended with YE, DL-vitamin mix, or glucose. Controls with no amendment did not lead to degradation and are not presented.

	Amendment *	OD600	Time (Days)
TBNPA	[1] Yeast extract	0.066	4
	[1] Vitamin mix	0.021	30–60
	[2] Glucose	0.089 ± 0.0049	4–5
DBNPG	[1] Yeast extract	0.079	4
	[1] Vitamin mix	0.020	30–60
	[2] Glucose	0.100 ± 0.0007	4–5
TBNPA and DBNPG	[3] Yeast extract	0.069 ± 0.0119	7
	[1] Vitamin mix	0.023	30–60
	[2] Glucose	0.087 ± 0.0070	4–5

[1] One replicate value; [2] two replicate average; [3] three replicate average. * Amendment concentrations: yeast extract 100mg L^{-1}; vitamin mix 2mL L^{-1}; glucose-100 mg $L^{-1.}$

Degradation rates were tested with the four-strain consortium with a mixture of TBNPA and DBNPG and yeast extract (100 mg L^{-1}). The separate and mixed substrate additions resulted in different degradation rates (Figure 2) (experiments were done in parallel). Separately, both compounds were degraded at a similar rate within 3–4 days (for TBNPA, $k_{TBNPA} = -0.994\ h^{-1}$, $R^2 = 0.9$; for DBNPG, $k_{DBNPG} = -0.972\ h^{-1}$, $R^2 = 0.9$). When incubated concurrently, full degradation was reached in 7 days ($k_{TBNPA} = 0.348 \pm 0.005\ h^{-1}$, $R^2 = 0.95$; $k_{DBNPG} = 0.333 \pm 0.004\ h^{-1}$, $R^2 = 0.96$) (Figure 2). A similar observation was observed with the DL-vitamin mix (data not shown). In both separate and concurrent experiments, the final concentration of either compounds or both together was 100 mg L^{-1}.

Simultaneous utilization of substrate mixtures can occur through enzymatic activity with broad substrate specificity [49]. In this case, co-metabolism is not relevant, as the simultaneous presence of both substrates is not required for degradation to occur. The simultaneous utilization of DBNPG and TBNPA at the same rate indicates a similar degradation mechanism for both compounds, such as the degradation of different length n-alkane by *Pseudomonas aeruginosa* DN1 [50] or methyl tert-butyl ether (MTBE) and n-alkanes by *Pseudomonas citronellolis* UAM-Ps1 [47].

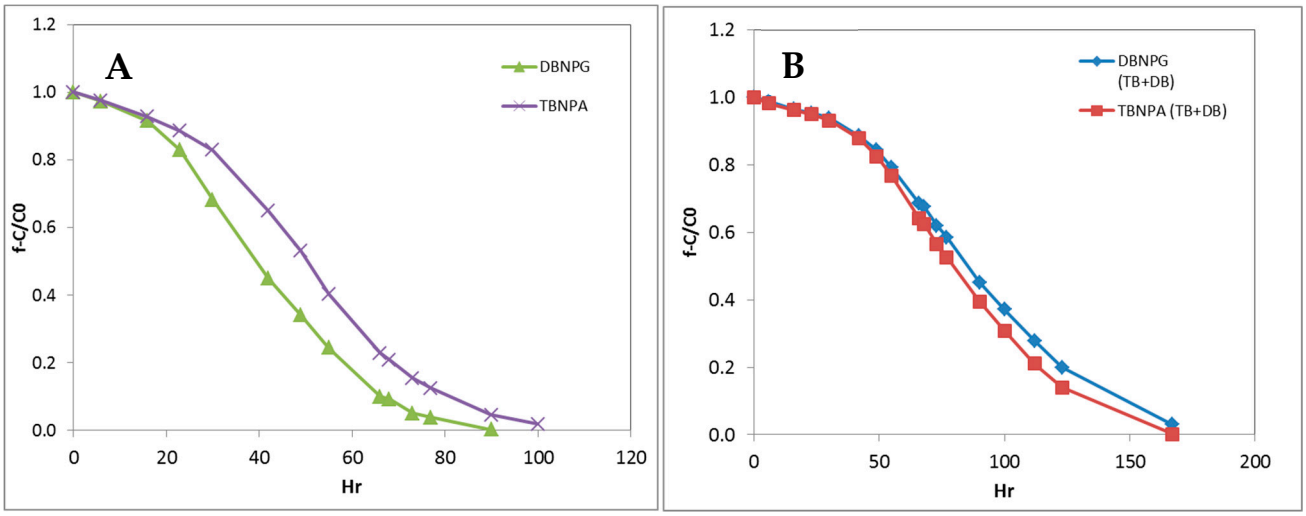

Figure 2. Microbial degradation during (**A**) separate (100 mg L^{-1} each) and (**B**) concurrent (50 mg L^{-1} each) supplement of DBNPG and TBNPA with the four-strain consortium and yeast extract amendment (Table 1). For (**A**) Each point represents one triplicate; (**B**) each point represents 3 replicates with standard deviation error bars.

Simultaneous utilization of substrate mixtures is often accompanied by a decrease in degradation rates in relation to rates obtained when supplemented separately [51]. This phenomenon is due to a competitive inhibitory effect. In this study, competitive inhibition was observed, while TBNPA and DBNPG degradation rates decreased in the mixed substrate experiments. Various BTEX mixtures exhibited similar processes [19] during the biodegradation of toluene and phenol by *Burkholderia* sp. JS150 A [24] and in cis-dichloroethene (cDCE) degrading microcosms, where the addition of different substrates led to decreased cDCE degradation rates [21,52].

3.3. Isotope Fractionation

A dual carbon–bromine isotope analysis was applied for the degradation of both compounds by the four-strain consortium. Significant isotope effects of carbon were observed for both compounds, with no detectable enrichment in bromine. The undetected bromine isotope enrichment implies that DBNPG, as was already shown for TBNPA, is not dehalogenated as the rate-limiting step of the reaction, despite the observable stoichiometric release of bromide to the solution [36,38]. In addition, DBNPG was not identified during HPLC analysis as a daughter product of TBNPA during biodegradation. TBNPA and DBNPG carbon enrichment factors were determined from the Rayleigh plot, with $\varepsilon = -8.0 \pm 0.2‰$ for TBNPA and $\varepsilon = -7.0 \pm 0.4‰$ for DBNPG (Figure 3). Similarly, the carbon and bromine isotope fractionation observed for the two compounds may indicate that the degradation occurs by the analogous pathway. Nevertheless, the slightly lower carbon isotopic effect during DBNPG degradation may be the result of slight masking in transport due to its more hydrophilic nature than TBNPA (2 hydroxyl groups in relation to 1).

The results suggest that the four-strain consortium is responsible for the degradation in the original enrichment culture while following a similar mechanism and with the same enzymes. This is based on the similarity between the carbon enrichment factors seen in this study and in Balaban et al., 2016, in which the original enrichment culture was examined [37]. In the initial enrichment, it was established that TBNPA and DBNPG degradation proceeded by a monooxygenase enzyme. Therefore, it is insinuated that this is the degradation mechanism of the four-strain consortium. Nevertheless, further inquiry to identify the enzyme in the four-strain consortium was not successful.

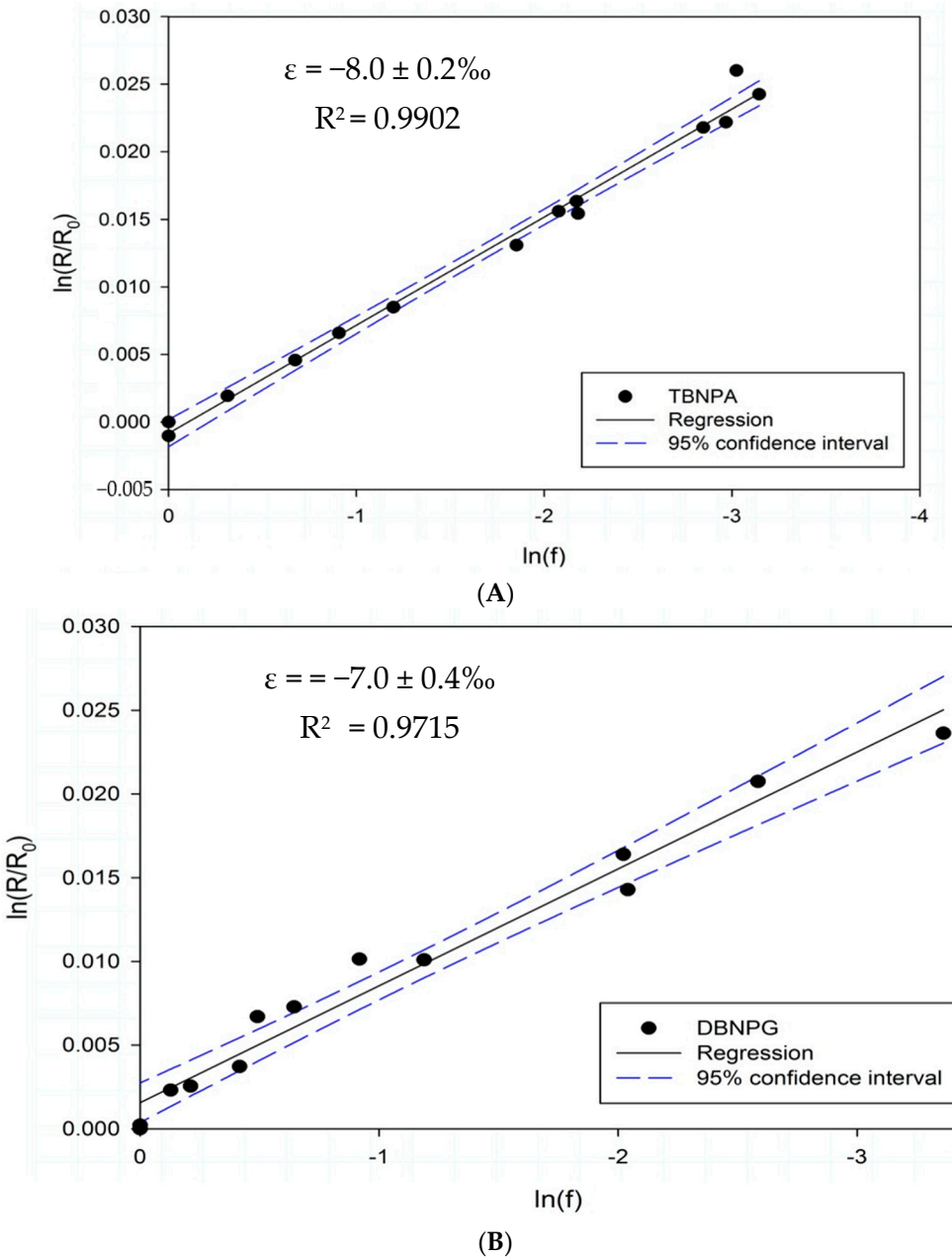

Figure 3. Rayleigh plot for carbon isotopes for (**A**) TBNPA aerobic biodegradation, (**B**) DBNPG aerobic biodegradation. f = fraction of degradation; Rt and Rt_0, carbon isotope composition of the compound at time t and zero, respectively.

4. Conclusions

In this study, the biodegradation of two brominated flame retardants, TBNPA and DBNPG, in aerobic conditions by a four-strain microbial consortium was demonstrated, seemingly through a monooxygenase mechanism. The presence of all four strains and a carbon amendment were mandatory to initiate degradation; this helps to implement a metabolic complementation connection between the strains. Based on the similarity of isotopic fractionation results to those received for the original enrichment culture, it can be understood that the four-strain consortium represents the actual degrading microbial group from the extensive original enrichment community. As TBNPA and DBNPG were found not to sustain microbial growth, carbon was a limiting factor for degradation. During the concurrent addition of TBNPA and DBNPG, simultaneous degradation occurred at a

slower rate (about half) than when added separately, suggesting an analogous monooxygenase mechanism.

The study's importance has two venues: The first is the establishment and culture of a limited consortium representative of an extensive environmentally enriched consortium. The second is the use of isotopic tools to ensure that the degrading isolated strains are representative of the original enrichment consortium. Further study of the four-strain consortium can allow us to investigate the degradation processes of brominated and halogenated compounds.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11146263/s1, Vitamin Mix, Strain Screening, Screening Media, DBNPG or TBNPA Degradation Screening Experiments, TBNPA Isolates screening, Degradation of DBNPG or TBNPA, Degradation rate and efficiency of TBNPA and DBNPG by the four-strain consortium, DB2-DB5, Discussion.

Author Contributions: Conceptualization, N.B., F.G., A.B. and Z.R.; methodology N.B. and F.G.; investigation, N.B. and A.A.T.; formal analysis, N.B., F.G., A.B. and Z.R.; visualization, N.B. and A.B.; writing—original draft preparation, N.B.; writing—review and editing, N.B., F.G., A.B., S.L.W. and Z.R. All authors have read and agreed to the published version of the manuscript.

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