Vietnam Journal of Science and Technology ***63*** *(3) (2025) 563-575*

doi:10.15625/2525-2518/18876

Isolation of bacteria capable of degrading organophosphate flame retardants

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Received: 11 September 2023; Accepted for publication: 15 May 2024

Abstract. Organophosphate flame retardants (OPFRs), widely used in industrial applications, pose potential risks to human health due to environmental contamination. This study aimed to isolate and identify OPFR-degrading bacteria from wastewater samples collected from the To Lich River. Samples were enriched in a medium supplemented with OPFRs, resulting in the isolation of 10 bacterial strains. Based on 16S rRNA gene analysis, the isolates belonged to the genera *Achromobacter*, *Pseudomonas*, *Bordetella*, and *Rhizobium*. All strains showed OPFR-degrading capacity, but *Achromobacter* sp. BWTL6 and *Rhizobium* sp. BWTL7 exhibited the highest efficiency, degrading all five tested OPFRs. Notably, both strains degraded over 70 % of tris(1,3-dichloro-2-propyl) phosphate (TDCPP) at 10 mg/L within 7 days and utilized other OPFRs, including tris(2-chloroethyl) phosphate (TCEP), triethyl phosphate (TEP), tris(2-butoxyethyl) phosphate (TBEP), and trimethyl phosphate (TMP) , for growth, with removal rates ranging from 40 - 58 %. Additionally, *Bordetella* sp. BWTL3 degraded 61.5 % of TDCPP and 97.7 % of TBEP. These findings highlight the potential of *Achromobacter* and *Rhizobium* strains for bioremediation of OPFR-contaminated environments.

Keywords: *Achromobacter*, *Rhizobium*, tris(1,3-dichloro-2-propyl)phosphate (TDCPP), tris(2-butoxyethyl) phosphate (TBEP), tris(2-chloroethyl) phosphate (TCEP).

Classification numbers: 3.1.1.

1. INTRODUCTION

Organophosphorus Flame Retardants (OPFRs) are known as additives in the manufacture of plastics and flame retardants, commonly applied in construction materials, household products, and different industries [1, 2]. Due to their excellent fire-retardant properties, good physical and chemical properties, OPFRs are incorporated into flammable materials to prevent combustion and slow down the spread of flames after ignition [3]. OPFRs are commonly used extensively in the production of flame retardants, plasticizers, antifoaming agents, lubricants,

and hydraulic fluids in various industries, including plastics, furniture and decorative materials, building materials, textiles, and electronic equipment [4, 5]. OPFRs are considered emerging pollutants due to their increasing widespread use as flame retardants in recent years, following the phase-out of certain brominated flame retardants (polybrominated diphenyl ethers - PBDEs) due to their environmental persistence, biotoxicity, and bioaccumulation [4]. Based on their different functional groups, organophosphate esters, which are chemical components of OPFRs, can be classified into halogenated and non-halogenated OPEs. Halogenated OPEs, such as tris(2-chloro-1-methyl ethyl) phosphate (TCPP), tris(1,3-dichloro-2-propyl)phosphate (TDCPP), and tris(2-chloroethyl) phosphate (TCEP), have been banned or restricted in products for children and residential upholstered furniture in the United States [6].

OPEs are incorporated into polymer materials through physical blending rather than chemical bonding, which makes them easily released into the surrounding environment through evaporation, abrasion, and dissolution [7]. Indeed, OPEs have been detected in a variety of environments, including air, water, sediment, dust, and soil [8]. In addition, OPEs have been detected in animals, plants, and even in hair and nails, urine, and breast milk [9, 10]. Prolonged exposure and accumulation of OPEs including TBEP, TnBP, triphenyl phosphate (TPhP), and other OPEs in the human body can cause various adverse effects, including nephrotoxicity, neurotoxicity, reproductive toxicity, carcinogenicity, and endocrine disruption [11, 12]. In the natural environment (water, air, and soil), chlorinated OPEs exhibit high persistence and low degradation capability [13-16]. Biological treatment is considered a suitable approach for effectively removing undesired OPEs, being cost-effective and environmentally friendly [17]. *Brevibacillus brevis* is known to have the capability to degrade TPhP and tricresyl phosphates [18].

In recent years, the rapid pace of urbanization and industrialization, coupled with a lack of effective waste control and treatment measures, especially in wastewater treatment plants, has resulted in water pollution in Ha Noi, particularly an increasing contamination of surface water with OPFRs [19]. Truong *et al*. (2013) determined the pollution of OPFRs such as TCEP, TCPP, TDCPP, TnBP, TBEP, TPhP, and diphenyl phosphate (DPhP) in surface water samples from the To Lich River, with concentrations ranging from 24 to 1950 ng/L. Therefore, the main objective of this study is to isolate effective microbes for bioremediation of OPFRs. This study also aims to provide new insights into the degradation efficiency of OPFRs by potential microbial strains.

2. MATERIALS AND METHODS

2.1. Materials

Two surface water samples (L1: 21°02'04" N, 105°48'21" E and L2: 21°02'26" N, 105°48'16" E) were collected from the To Lich River (Cau Giay district) in the Hanoi urban area (Viet Nam) to isolate bacterial strains capable of degrading OPFRs.

Seven standard OPFRs, including trimethyl phosphate (TMP), triethyl phosphate (TEP), tris(2-ethylhexyl) phosphate (TEHP), TBEP, TPhP, TCEP, and tris(1,3-dichloro-2-propyl)phosphate (TDCPP), were supplied by Dr. Ehrenstorfer (LCG, Germany). Surrogate standards (TEP-d15, phosphoric acid tripropyl ester-d21, phosphoric acid tributyl ester-d27, triphenyl phosphate-d15, and tris(2-ethylhexyl) phosphate-d51) were provided by Toronto Research Chemicals Inc. (Canada). In this study, analytical grade methanol was supplied by Merck (Germany). All glassware was cleaned and rinsed with solvent before use. Methanol was used to prepare the mixed stock solution (containing all analytes at a concentration of 4000

mg/L) and the standard curve solution (1 - 100 µg/L) used for analyzing OPFR concentrations. The prepared mixed stock solution was stored at −20 °C.

2.2. Methods

2.2.1. Sampling method

Water samples were collected in dark glass bottles. All sampling equipment was pre-rinsed with ethanol, distilled water, and acetone before use and then rinsed three times with water samples before collection. Single-use gloves were used during the sampling process. The collected samples were stored at 4 °C for further studies.

2.2.2. Isolation of bacteria capable of degrading OPFRs from water and sediment samples collected from the To Lich River

Ten grams of wastewater samples collected from the To Lich River (Cau Giay district, Ha Noi, Viet Nam) were transferred into a 250 mL Erlenmeyer flask containing 90 mL of modified A-Cl medium. The medium consisted of glucose 10 g/L, $(NH_4)_2SO_4$ 1 g/L, $MgSO_4.7H_2O$ 0.2 g/L, $Ca(NO_3)_2.4H_2O$ 0.032 g/L, yeast extract 0.5 g/L, and 1 mL/L of trace element solution, with a pH of 7. The trace element solution contained 500 mg $FeSO_4.7H_2O$, 143 mg $MnSO_4.2H_2O$, 22 mg $ZnSO_4.7H_2O$, 12 mg $CoSO_4.7H_2O$, 3 mg $CuSO_4.5H_2O$, 2.3 mg $Na_2WO_4.2H_2O$, and 2 mg $Na_2MoO_4.2H_2O$ dissolved in 1 liter of distilled water [20]. The enrichment culture samples were simultaneously supplemented with seven OPFRs including TMP, TEP, TEHP, TBEP, TPhP, TCEP, and TDCPP (10 mg/L of each OPFR). These compounds were added as a source of carbon and phosphorus in the culture flasks, which were then shaken at 150 rpm at 30 °C for 10 days. After 10 days, the 1st enriched culture samples (10 % v/v) were transferred to Erlenmeyer flasks containing 90 mL of fresh modified A-Cl medium supplemented with the OPFRs. The cultures were incubated under the same conditions as mentioned above. After another 10 days, 10 mL of these cultures (2nd enrichment) were then transferred into 90 mL of fresh modified A-Cl medium added with OPFRs described above. The 3rd enrichment steps were carried out in the same way as the 2nd enrichment steps. Finally, the 3rd enriched culture was spread onto A-Cl agar plates containing 2 mg/L of each OPFR as the sole phosphorus source. The A-Cl agar plates were then incubated at 30 °C until colonies formed. After pure-colony isolation, the isolated bacteria were stored at −80 °C in 30 % glycerol.

2.2.3. Bacteria identification

The total DNA of the bacterial strains was extracted using an ABT kit (ABT Solutions LLC, Viet Nam) following the manufacturer's instructions. A partial sequencing of the 16S rRNA gene with a size of approximately 1500 bp was amplified using the primer pair 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1512R (5′-ACGGYTACCTTGTTACGACTT-3′) [21] as described previously. Reactions were performed in a PCR G-STORM (England). PCR products were purified and sequenced by Macrogen Europe. These sequences were compared to known 16S rRNA gene sequences available on the GenBank database using BLAST searches at the National Center for Biotechnology Information (https://blast.ncbi. nlm.nih.gov/Blast.cgi). The sequences were aligned and a phylogenetic tree was visualized using the MEGA version 7 software by the neighbor-joining method [22]. The nucleotide sequences of the isolated bacterial strains were registered on the NCBI gene bank. The nucleotide sequences of the partial 16S rRNA gene for the 10 bacterial strains BWTL1, BWTL2, BWTL3, BWTL4, BWTL5, BWTL6,

BWTL7, BWTL8, BWTL9, and BWTL10 have been deposited in GenBank under accession numbers ranging from OR481672 to OR481681.

2.2.4. Degradation of OPFRs by isolated bacterial strains

Isolated bacterial strains were inoculated into A-Cl medium and shaken at 150 rpm, 30 ^{o}C for 24 hours to increase biomass growth. Subsequently, the cell culture broth was centrifuged (6000 rpm for 3 min), and washed three times with A-Cl medium. The bacterial culture supernatant (OD600 ~ 0.2) was added to a 100 mL Erlenmeyer flask containing 40 mL of A-Cl medium supplemented with 10 mg/L of each OPFR as the sole phosphorus source to evaluate the degradation of OPFRs in 7 days of cultivation. Control sample contains only medium and OPFRs at the same concentrations. The experiments were conducted in triplicate. Culture samples were taken on days 0, 1, 3, 5 and 7 to determine the concentrations of OPFRs and cell growth rates at OD 600 nm. The optical density at a wavelength of 600 nm (OD600) of the culture medium was measured using a Hitachi UV-2900 spectrophotometer (Hitachi, Japan) to determine bacterial density.

2.2.5. Quantification of OPFRs

A UHPLC system (UltiMate 3000+, Thermo Scientific, MA, USA) combined with an MS/MS spectrometer (API 3200, Applied Bio-System/MDS SCIEX, USA) were used to analyze the OPFR group in this study. An Acclaim Mixed-Mode HILIC column (2.1 mm × 150 mm, 5 µm, Thermo Fisher) was selected to separate the target analytes. The column chamber temperature was maintained at 35 °C. The mobile phase consisted of two channels, channel A (ultrapure water) and channel B (acetonitrile). The mobile phase flow rate was 0.3 mL/min. The solvent gradient program was illustrated as follows: hold 30 % channel B for the first 5 min, increase linearly to 70 % channel B at 8 min, increase rapidly to 100 % channel B at 13 min, then gradually reduce to only 30 % channel B at 15 min, maintain up to 20 min before the next sample injection. In an MS/MS detector, the positive electrospray ionization [23] mode and multiple reaction monitoring (MRM) mode were employed. The optimal key parameters included ion spray voltage of 5000 V, collision gas pressure of 0.02 MPa, sheath gas pressure of 0.18 MPa and auxiliary gas pressure of 0.22 MPa. The source temperature was set at 400 °C. TraceFinder 4.0 software was applied to process the data set.

2.2.6. Statistical analysis

Data and statistical analyses were performed using Excel 2013 and GraphPad Prism 8.0.2. All of the experiments were performed in triplicate, and the data are expressed as means ± standard deviation. One-way analysis of variance (ANOVA) was used to determine the significant differences between values, followed by Dunnett's T3 multiple comparison test. A probability of $p \leq 0.05$ was considered significant.

3. RESULTS AND DISCUSSION

3.1. Isolation and selection of bacteria capable of degrading OPFRs

From wastewater samples collected in the To Lich River, bacterial strains capable of utilizing OPFRs were isolated by the enrichment method on A-Cl medium supplemented with

OPFRs. Microbial consortia grown on agar plates were then separated into pure strains, and bacterial morphology was observed.

Ten microbial strains isolated on agar plates have different colony morphologies. The majority of colonies were morphologically similar and could be divided into four main groups according to diameter: group with diameters of 1 - 1.5 mm has a round shape, smooth border, slightly convex surface, and creamy-white color (denoted as BWTL1, BWTL3); group with diameters of 2 - 4 mm has a round shape, smooth surface, slight convexity and milky to creamy yellow color (denoted as BWTL4, BWTL6, BWTL7, BWTL8); group with diameters of 2 - 5 mm has a round shape, flat and matte surface with irregular, creamy-yellow edges (denoted as BWTL2, BWTL10); and group with diameters of 2 - 6 mm has a round shape, slightly rough, flat, sparkling surface, and a cloudy creamy-white color (denoted as BWTL5, BWTL9).

3.2. Classification of isolated bacterial strains based on 16S rRNA gene sequence

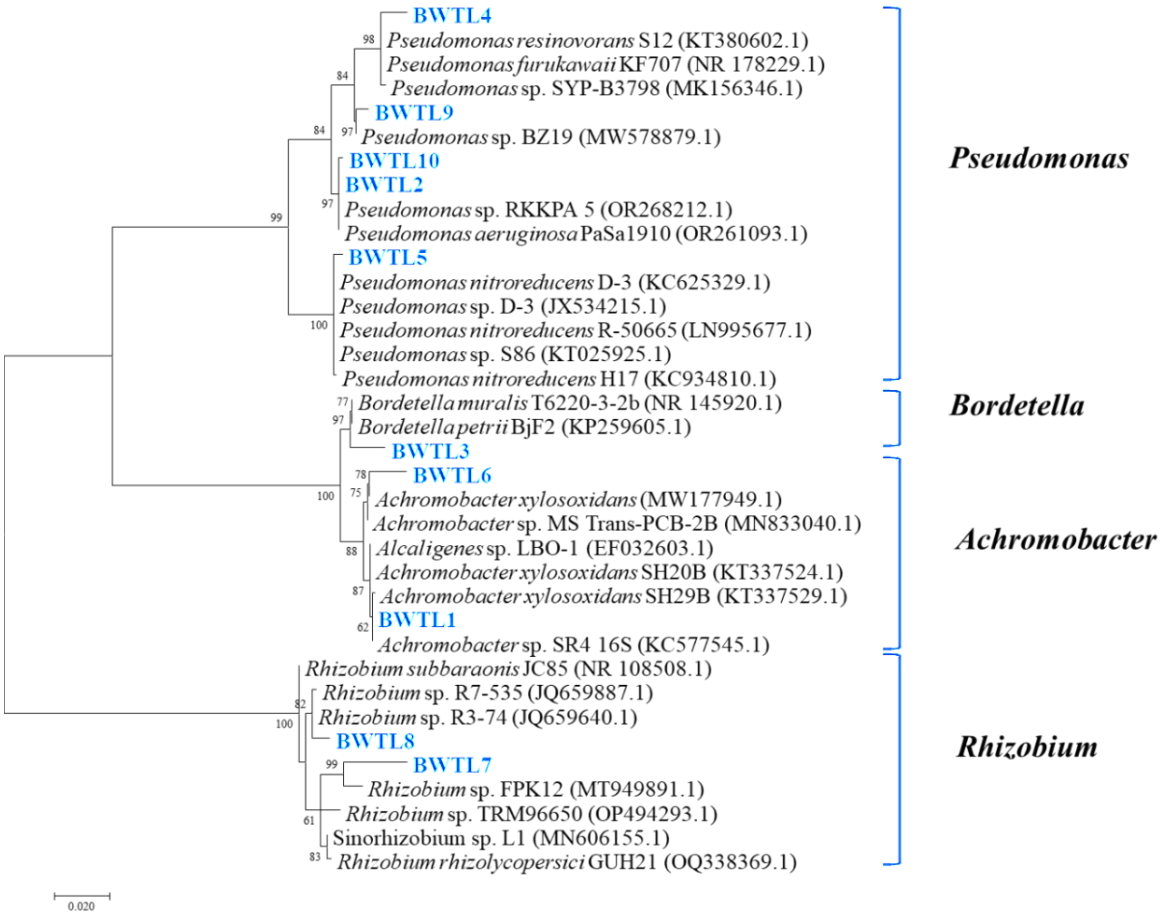

Figure 1. Phylogenetic tree of isolated bacterial strains. Bootstrap values greater than 50 % are displayed at the nodes.

In Figure 1, strains BWTL2, BWTL4, BWTL5, BWTL9, and BWTL10 cluster closely, indicating a genetic relationship with the genus *Pseudomonas*. BLAST analysis shows 99 % similarity with species such as *Pseudomonas* sp. RKKPA 5 and *P. aeruginosa* PaSa1910, so they

are named *Pseudomonas* sp. BWTL2, *Pseudomonas* sp. BWTL4, *Pseudomonas* sp. BWTL5, *Pseudomonas* sp. BWTL9, and *Pseudomonas* sp. BWTL10. Strain BWTL3 shares 98 % similarity with *Bordetella petrii* and *B. muralis*, and is identified as *Bordetella* sp. BWTL3. Strains BWTL1 and BWTL6 show 99 % and 98 % similarity with *Achromobacter xylosoxidans*, thus are named *Achromobacter* sp. BWTL1 and *Achromobacter* sp. BWTL6. Finally, strains BWTL7 and BWTL8 share 99 % similarity with *Rhizobium* sp. FPK12 and R3-74 and are designated *Rhizobium* sp. BWTL7 and *Rhizobium* sp. BWTL8. These identifications are supported by both 16S rRNA sequence similarity and phylogenetic analysis.

Microbial biodegradation are often employed to remediate environments contaminated with OPFRs. Bacterial strains belonging to genera such as *Brevibacillus*, *Sphingomonas*, *Sphingopyxis*, *Rhodococcus* can degrade some OPFRs through biodegradation processes. Specifically, the genus *Pseudomonas* has been studied and confirmed to be able to utilize TnBP as a phosphorus source for its growth and the degradation of OPFRs [24, 25]. The identification of the 10 bacterial strains, isolated from water samples contaminated with OPFRs from the To Lich River in Ha Noi, suggests their potential utility in the remediation of environments polluted with OPFRs.

3.3. Degradation of OPFRs by isolated bacterial strains

The growth of 10 isolated bacterial strains on A-Cl medium supplemented with 10 mg/L of each OPFR is shown in Figure 2. After 7 days, all strains exhibited increased biomass, with OD_{600} values ranging from 0.432 to 1.718. *Achromobacter* sp. BWTL6 showed the highest growth (OD_{600} = 1.718), followed by *Bordetella* sp. BWTL4 and *Rhizobium* sp. BWTL7 (OD_{600} > 1). In contrast, *Pseudomonas* sp. BWTL3 and BWTL9 displayed minimal growth. These findings suggest that several strains can grow effectively using OPFRs as the carbon and phosphorus sources, highlighting their potential for bioremediation applications.

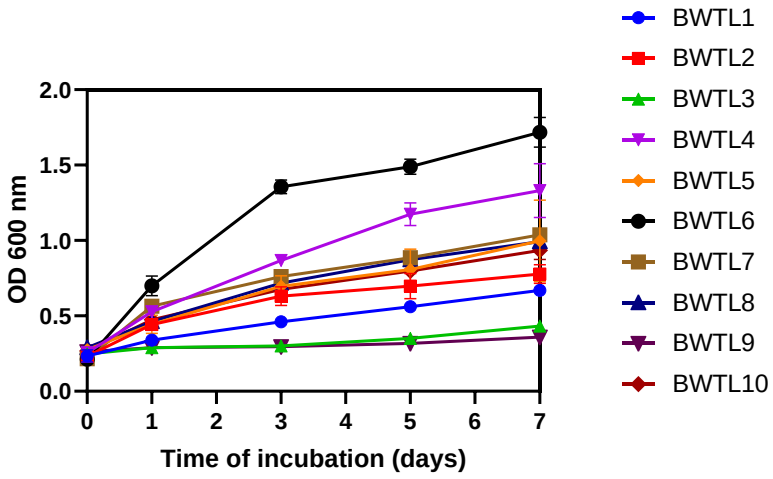

Figure 2. Growth of isolated bacterial strains on A-Cl medium supplemented with OPFRs.

In the degradation process of individual OPFRs, Hou *et al*. (2021) observed a significant increase in microbial biomass with optical density measured at 600 nm gradually increasing from 0.1 to 2.5 [26]. The results of our study are similar to this study, showing the growth and

development of bacterial strains on A-Cl medium supplemented with OPFRs after 7 days of cultivation. During the microbial growth, microorganisms gradually adapt and utilize OPFRs as carbon and phosphorus sources for their survival. In the metabolic system, easily degradable substrates such as glucose are preferentially used as energy and carbon sources for microbial growth before OPFRs provide sufficient carbon. Consequently, co-metabolism of non-halogenated OPFRs and external organic sources may facilitate the degradation of these pollutants.

A total of 10 bacterial strains were isolated from wastewater samples of the To Lich River, enriched on A-Cl medium supplemented with organophosphate flame retardants (OPFRs). The degradation efficiency of seven OPFRs (each at 10 mg/L) by these isolates after 7 days of cultivation is presented in Figure 3 and Table 1. Additionally, UHPLC-MS/MS analysis (Figure 4) compared mass spectra of untreated controls and samples treated with *Bordetella* sp. BWTL3, *Achromobacter* sp. BWTL6, and *Rhizobium* sp. BWTL7. In the control samples, partial degradation of TPhP and TEHP was observed, with degradation efficiencies of 40.3% and 59%, respectively, suggesting chemical instability of these compounds (Table 1).

Table 1. Degradation efficiency of OPFRs by isolated bacterial strains after 7 days of cultivation.

Sample	Degradation efficiency of OPFRs (%)						
	TMP	TEP	TCEP	TDCPP	TPhP	TBEP	TEHP
Control	0	5.3	0	5.8	40.3	5.8	59.0
BWTL1	30.1^{c}	24.7^{c}	24.5^{c}	56.8^{c}	59.3^{c}	28.8^{c}	51.3
BWTL2	21.6^{c}	18.0^{a}	12.9^{b}	56.9^{c}	62.5^{c}	12.3	93.9^{c}
BWTL3	0	4.0	14.8^{c}	61.5^{c}	68.2^{c}	97.7^{c}	74.9^{c}
BWTL4	4.3	4.5	1.0	30.7^{c}	52.8^{a}	1.6	60.5
BWTL5	15.6^{c}	3.3	7.9	48.4^{c}	51.3^{a}	2.4	64.2
BWTL6	40.3^{c}	43.6^{c}	53.1^{c}	70.6^{c}	51.7^{a}	55.4^{c}	78.8^{c}
BWTL7	53.3^{c}	53.8^{c}	58.5^{c}	74.4^{c}	82.4^{c}	58.1^{c}	92.6^{c}
BWTL8	9.1	13.5	25.8^{c}	69.5^{c}	77.3^{c}	23.6^{c}	89.5^{c}
BWTL9	2.4	2.2	24.0^{c}	30.1^{c}	36.6	9.8	34.8^{c}
BWTL10	2.6	8.2	20.5^{c}	52.3^{c}	76.3^{c}	18.6^{b}	91.1^{c}

In the bacterial cultures, degradation efficiency increased over time, reaching its peak on day 7. Several strains, including *Bordetella* sp. BWTL3, *Pseudomonas* sp. BWTL4, *Pseudomonas* sp. BWTL5, *Pseudomonas* sp. BWTL9, and *Pseudomonas* sp. BWTL10, showed poor degradation of TMP and TEP. Likewise, *Pseudomonas* sp. BWTL2, *Pseudomonas* sp. BWTL4, *Pseudomonas* sp. BWTL5, *Pseudomonas* sp. BWTL9, and *Pseudomonas* sp. BWTL10 exhibited low degradation of TBEP and TCEP. Degradation of TDCPP ranged from 30 % to 74 % among all strains. Notably, *Achromobacter* sp. BWTL6 and *Rhizobium* sp. BWTL7 demonstrated the highest degradation efficiency, removing over 70 % of TDCPP and significantly degrading TCEP, TEP, TBEP, and TMP (One-way ANOVA, $p \leq 0.0001$). These two strains also showed strong growth, indicating their potential for OPFR bioremediation.

Bordetella sp. BWTL3 degraded 61.5 % of TDCPP and 97.7 % of TBEP. In contrast, *Pseudomonas* sp. BWTL4 and *Pseudomonas* sp. BWTL9 exhibited the lowest degradation capabilities and poor growth, possibly due to OPFR toxicity. The degradation of TMP, TEP, TCEP, and TBEP by the remaining isolates were all below 20 % (Table 1).

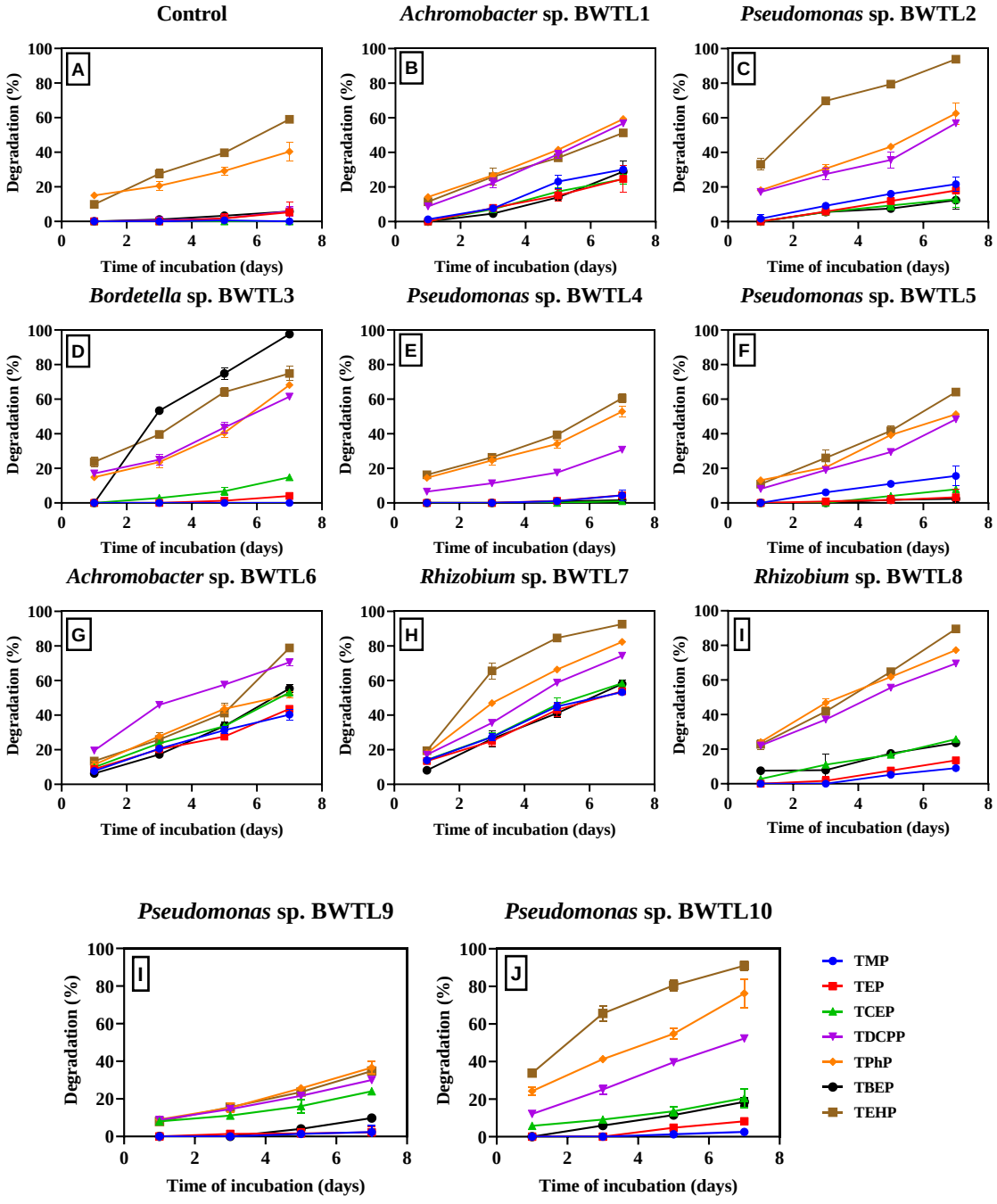

Figure 3. Degradation of OPFRs by isolated bacterial strains (A-J) after 7 days of cultivation.

Table 2. Analyte specific MS/MS parameters obtained in the positive electrospray ionization mode.

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	Collusion Energy (eV)	Retention Time (min)
TMP	141	79, 109	22, 16	2.97
TEP	183	99, 127	18, 20	4.74
TCEP	285	63, 99	22, 20	5.63
TDCPP	431	99, 209	26, 13	12.12
TPhP	327	77, 152	40, 40	12.78
TBEP	399	199, 299	17, 15	14.58
TEHP	435	99, 113	20, 13	21.47

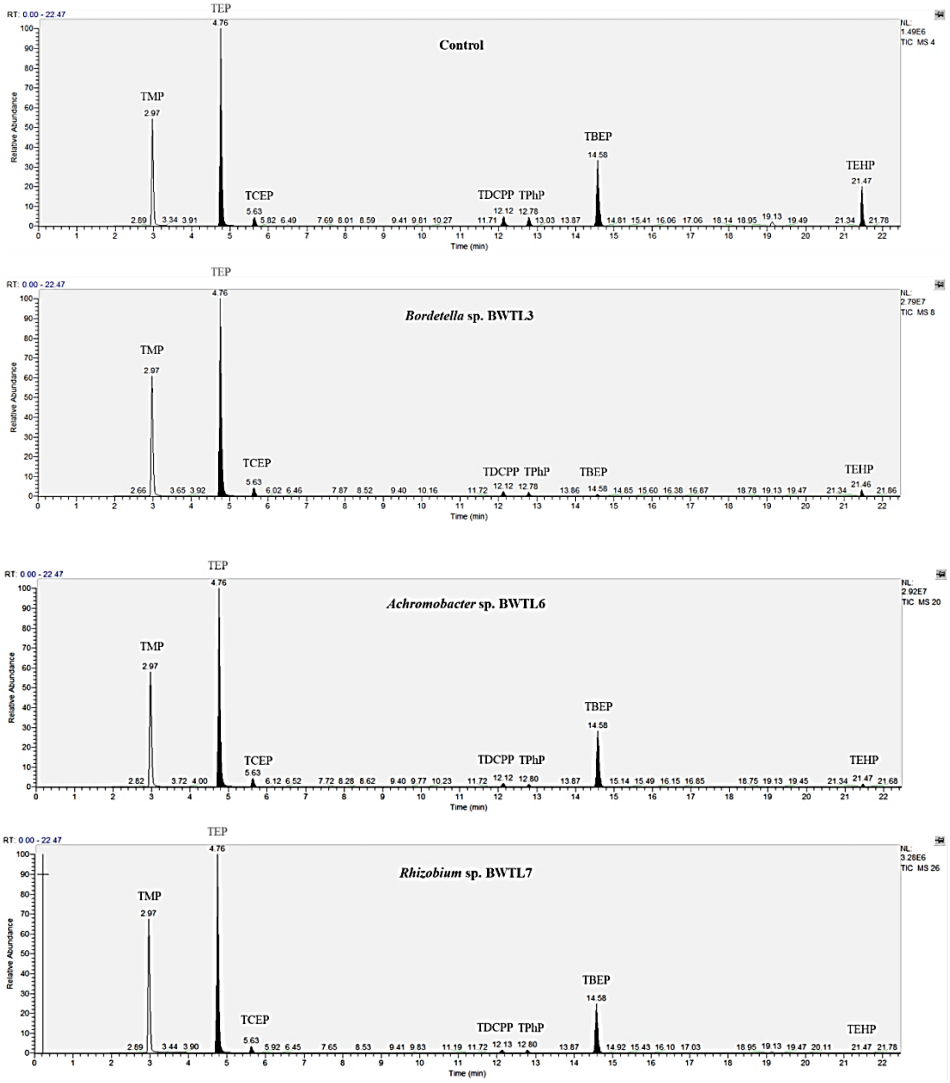

Figure 4. Mass spectra obtained by UHPLC-MS/MS analysis for control sample and bacterial treatment (*Bordetella* sp. BWTL3, *Achromobacter* sp. BWTL6 and *Rhizobium* sp. BWTL7) after 7 days of cultivation.

The letters a, b, c indicate significant differences in degradation efficiency of OPFRs compared with uninoculated control at $p \leq 0.05$, 0.001, 0.0001, respectively (One-way ANOVA).

Table 2 shows specific MS/MS parameters obtained in the positive electrospray ionization mode. The results indicate that retention time of OPFRs ranges from 2.97 to 21.47 minutes.

Current research on OPFR-degrading microorganisms remains limited, with only a few strains from genera such as *Roseobacte*r, *Sphingomonas*, *Sphingobium*, *Brevibacillus*, *Rhodococcus*, and *Sphingopyxis* being reported [20, 27, 28]. Previous study of Wang et al. (2019) showed that *Rhodococcus* sp. YC-JH2 and *Sphingopyxis* sp. YC-JH3 could degrade up to 96.2% of TPhP within 7 days, while *Roseobacter* strain YS-57 removed over 99 % of 0.5 mg/L TPhP in 3 days without glucose [27]. However, in our study, TPhP and TEHP were partially degraded abiotically (40.3 % and 59 %, respectively, after 168 hours). Additionally, *Sphingomonas* sp. TDK1 and *Sphingobium* sp. TCM1 degraded 100 % of TCEP and TDCPP within 6 hours [20]. Our findings demonstrate, for the first time, that strains *Bordetella* sp. BWTL3, *Achromobacter* sp. BWTL6, and *Rhizobium* sp. BWTL7 can degrade all five tested OPFRs, suggesting their potential for bioremediation in OPFR-contaminated environments in Viet Nam.

4. CONCLUSIONS

Ten bacterial strains from the genera *Pseudomonas*, *Bordetella*, *Achromobacter*, and *Rhizobium* were isolated from wastewater samples collected from the To Lich River, Ha Noi, Viet Nam and showed varying abilities to degrade OPFRs. Notably, *Achromobacter* sp. BWTL6 and *Rhizobium* sp. BWTL7 efficiently degraded all five tested OPFRs (TDCPP, TCEP, TEP, TBEP, and TMP), highlighting their strong potential for bioremediation. This study is the first to report OPFR degradation by strains of *Achromobacter*, *Bordetella*, and *Rhizobium*, expanding the known diversity of OPFR-degrading bacteria.

Acknowledgments. This research was supported by funding from the Basic Research Project with code VHH.2023.10, Institute of Chemistry, Vietnam Academy of Science and Technology.

CRediT authorcontribution statement. Thi Lan Anh Nguyen: Conceptualization, Methodology, Formal analysis, Investigation, Writing-Original draft preparation. Thi Phuong Pham: Methodology, Formal analysis, Investigation. Thi Thu Hien Tran: Methodology. Khac Hieu Phung: Methodology. Thi Thu Lan Tran: Reviewing and Editing. Hai Yen Dao: Reviewing and Editing.

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.

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