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Unraveling metabolic flexibility of rhodococci in PCB transformation

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Abstract:	Even though the genetic attributes suggest presence of multiple degradation pathways, most of rhodococci are known to transform PCBs only via regular biphenyl (bph) pathway. Using GC-MS analysis, we monitored products formed during transformation of 2,4,4'-trichlorobiphenyl, 2,2',5,5'-tetrachlorobiphenyl and 2,4,3'-trichlorobiphenyl by previously characterized PCB-degrading rhodococci Z6, T6, R2, and Z57, with the aim to explore their metabolic pleiotropy in PCB transformations. A striking number of different transformation products (TPs) carrying a phenyl ring as a substituent, both those generated as a part of the bph pathway and an array of unexpected TPs, implied a curious transformation ability. We hypothesized that studied rhodococcal isolates, besides the regular one, use at least two alternative pathways for PCB transformation, including the pathway leading to acetophenone formation (via 3,4 (4,5) dioxygenase attack on the molecule), and a third sideway pathway that includes stepwise oxidative decarboxylation of the aliphatic side chain of the 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate. Structure of the identified chlorinated benzoic acids and acetophenones allowed us to hypothesize that the first two pathways were the outcome of a ring-hydroxylating dioxygenase with the ability to attack both the 2,3(5,6) and the 3,4(4,5) positions of the biphenyl ring as well as dechlorination activity at both, -ortho and -para positions. We propose that several TPs produced by the bph pathway could have caused the triggering of the third sideway pathway. In conclusion, this study proposed ability of rhodococci to use different strategies in PCB transformation, which allows them to circumvent potential negative aspect of TPs on the overall transformation pathway.
Opposed Reviewers:	

Unraveling metabolic flexibility of rhodococci in PCB transformation

Petrić, Ines[1*], Drevenkar, Vlasta[2], Fingler, Sanja[2], Begonja Kolar, Ana[3], Hršak, Dubravka[1], Martin-Laurent, Fabrice[4], Udiković-Kolić, Nikolina[1]

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Highlights (3 to 5 bullet points (maximum 85 characters including spaces per bullet point)

Unraveling metabolic flexibility of rhodococci in PCB transformation

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Highlights

1. Tested rhodococci use complex metabolic hub for PCB transformation
2. Rhodococci use regular *bph* pathway in PCB transformation
3. Alternative pathways works via production of acetophenones
4. Third pathway includes stepwise oxidative decarboxylation of the aliphatic side chain of the HOPDA
5. Rhodococci harbor specific ring-hydroxylating dioxygenase with dechlorination ability

Response to reviewers/editor in question & answer format (word file)

Respected Editor,

We thank both the reviewers and the Editor for considering our manuscript "Unravelling metabolic flexibility of rhodococci in PCB transformation" for publishing in the Chemosphere journal. We have carefully reviewed all the comments and changed the manuscript accordingly. All changes have been highlighted in the manuscript with yellow colour. New Figure 1 has been produced. Down below you can find answers to all of the reviewer's comments.

Best regards,

Ines Petrić,

On behalf of all co-authors

ANSWERS TO THE COMMENTS FROM EDITORS AND REVIEWERS

Reviewer #1: The manuscript investigated the biotransformation of three PCB congeners by microbes. The metabolites of PCBs were identified and the metabolism pathways of PCBs were discussed. The manuscript provided useful information on microbial metabolism of PCBs. Minor revisions were suggested.

Comment 1: The chosen PCB congeners were not the main PCBs in environment, why not study the metabolism of indicator PCBs or dioxin-like PCBs?

Answer: *Actually, both 2,4,4'-trichlorobiphenyl (PCB-28) and 2,2',5,5'-tetrachlorobiphenyl (PCB-52), studied in this manuscript, are the PCB indicator congeners. According to the IUPAC (International Union of Pure and Applied Chemistry) numbering system, the numbers of seven indicator* ***PCBs*** *are #28, 52, 101, 118, 138, 153, and 180. So yes, we did study indicator PCBs within this study. To make this clear, we have added the IUPAC numbers of the studied PCB congeners (PCB-28, PCB-52, PCB-25) in the manuscript Abstract (line 19) and under manuscript Introduction, lines 128 and 129.*

Likewise, since the focus of our study was on PCB transformation pathways, rather than on the PCB environmental relevance, and we were driven by the idea to study transformation of congeners with blocked 2,3 and 3,4 position of the biphenyl ring, we decided to include in our study those congeners having different chlorination patterns. Consequently, our priory focus was not on dioxin-like congeners.

Comment 2: the quality assurance and quality control of PCB analysis were missing in the text. For instance, the name and amount of internal standard, surrogate standard, the recoveries of target PCBs in spiked samples or standard reference materials, and the relative standard deviations of replicate samples.

Answer: *We thank the reviewer for this comment. However, this paper represents a first "screening study" in which we focused not on the quantity of the produced transformation products but rather on the qualitative analysis, i.e. on identification as many as possible PCB transformation products. Consequently, internal standard or surrogate standard were not added. As you can see in the text we do not mention recoveries or quantity of either spiked PCBs neither observed transformation products.*

Comment 3: Plenty of PCB metabolites were detected after metabolism assay, were these metabolites identified in authentic environmental samples, such as soil and sediment?

Answer: *Thank you for this comment. We are not aware of the fact that such and similar transformation products (TP) have been detected in authentic environmental samples and this is something that we have planned for our future research. However, this could be problematic for several reasons. First, our experiment was done in the controlled conditions with only one congener added. In comparison, in the nature, PCBs are found in mixtures and therefore it would be difficult to follow which TP is produced from which congeners. Likewise, toxicological effect of other congeners present in these mixtures could interrupt degradation to proceed in this way. Finally, due to lower amounts of PCBs found in natural*

environment, when compared in the controlled experiment, we expect to find in the environment even lower amount of produced TP, making detection and identification more challenging. However, since all of our rhodococci tested here are originating from soil and sea environment we believe that similar transformation pathway should exist in their authentic environment.

Comment 4: Is it possible to know the specific enzymes mediating different biotransformation pathways?

Answer: Thank you for this comment. This is a very interesting remark and surely that would be exciting to find out. To do this we would need to settle the experiment in different way. One way is to follow the expression of selected genes (DNA) or enzymes (cDNA) by qPCR (real-time PCR) within different phases of the transformation reaction. Therefore, we would need to settle experiment in the way not to add congener itself in the reaction but to add specific TP and follow in parallel its transformation and expression of expected gene (DNA) or enzyme (cDNA). Unfortunately, in the laboratory we do not have expertise to do this type of analyses, however, this is a good point to think about.

Reviewer #2: The manuscript reports new degradation pathways of low chlorinated PCBs (3Cl-CB and 4Cl-CB) by rhodococcal isolates. The paper provides an important scientific information, which is of great interest to Chemosphere. The experimental findings are very interesting and discussed thoroughly.

Answer: *We thank the reviewer for his encouraging opinion on the results given within the manuscript.*

Comment1: I suggest to make a little bit larger in Fig. 1 the legend font because the enzyme names are not well readable.

Answer: *We have corrected Figure 1 according to the reviewers comment.*

Comment 2: Line 39: Remove perhaps at the start of the sentence e re-write as follows:

"PCBs, which comprise a family of 209 related compounds (congeners) and each with a distinct structure and environmental behaviour, are among the most notorius persistent organic pollutants (POPs) under the Stockholm convention (UNEP 2008).

Answer: *This sentence has been changed as suggested by the reviewer.*

Comment 3: Line 51: I suggest to write: "..contaminate environments" instead of .. "contaminate the environment"

Answer: *This sentence has been changed as suggested by the reviewer.*

Comment 4: line 113: I suggest: "known for its high metabolic versatility"

Answer: *This sentence has been changed as suggested by the reviewer.*

Comment 5: line 120: I suggest to write ..."pathway was considered" instead of ..." pathway was used"

Answer: *This sentence has been changed as suggested by the reviewer.*

Comment 6: line 71: Reference Erickson et al 1992 or 1993? Please, check some references:

line 115: Iwasaki et al 2007 or 2006?

Answer: *We thank the reviewer for noticing the mistakes in the reference. We have checked the year of publishing of the mentioned paper and changed the reference accordingly.*

line 89: Bedard et al 1990 or 1984 ?

Answer: *This reference was correctly written.*

Unraveling metabolic flexibility of rhodococci in PCB transformation

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Abstract

Even though the genetic attributes suggest presence of multiple degradation pathways, most of rhodococci are known to transform PCBs only via regular biphenyl (bph) pathway. Using GC-MS analysis, we monitored products formed during transformation of 2,4,4'-trichlorobiphenyl (PCB-28), 2,2',5,5'-tetrachlorobiphenyl (PCB-52) and 2,4,3'-trichlorobiphenyl (PCB-25) by previously characterized PCB-degrading rhodococci Z6, T6, R2, and Z57, with the aim to explore their metabolic pleiotropy in PCB transformations. A striking number of different transformation products (TPs) carrying a phenyl ring as a substituent, both those generated as a part of the bph pathway and an array of unexpected TPs, implied a curious transformation ability. We hypothesized that studied rhodococcal isolates, besides the regular one, use at least two alternative pathways for PCB transformation, including the pathway leading to acetophenone formation (via 3,4 (4,5) dioxygenase attack on the molecule), and a third sideway pathway that includes stepwise oxidative decarboxylation of the aliphatic side chain of the 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate. Structure of the identified chlorinated benzoic acids and acetophenones allowed us to hypothesize that the first two pathways were the outcome of a ring-hydroxylating dioxygenase with the ability to attack both the 2,3(5,6) and the 3,4(4,5) positions of the biphenyl ring as well as dechlorination activity at both, -ortho and -para positions. We propose that several TPs produced by the bph pathway could have caused the triggering of the third sideway pathway. In conclusion, this study proposed ability of rhodococci to use different strategies in PCB transformation, which allows them to circumvent potential negative aspect of TPs on the overall transformation pathway.

Keywords: polychlorinated biphenyl, biotransformation, *Rhodococcus*, *bph* pathway, transformation products, multiple pathways

1. Introduction

PCBs, which comprises a family of 209 related compounds (congeners) and each with a distinct structure and environmental behaviour, are among the most notorious persistent organic pollutants (POPs)under the Stockholm convention (UNEP 2008). Unfortunately, today`s common perception of PCBs as "legacy" POPs is very misleading. Despite the fact that their manufacture was banned almost five decades ago, PCBs are still entering the environment, mainly *via* point source pollution, inadequately managed hazardous waste, leaching from electrical equipment or during waste treatment. Due to their environmental persistence and known ability for long distance migrations by natural atmospheric and oceanic processes (Ge et al., 2013), they are found all over the globe. By recycling throughout the environment, they often reach crops, animals and humans with concurrent bioaccumulation and biomagnification in the food web (Turrio-Baldassarri et al., 2007). In recent years, it has been conclusively shown that the so-called "non-Aroclor PCBs" also contaminate environment. These "non-legacy" PCBs represent unintentional by-products of manufacturing processes of certain pigments used in dyes, inks, and paints but can be unintentionally produced from any chemical process that involves C, Cl and elevated temperatures or catalysts (Grossman, 2013; Rudel and Perovich, 2009). Studies imply a complex picture of how both "non-legacy" PCBs alongside with the persisting "legacy" PCBs affect the environment and human health (Grossman, 2013).

Among an array of possible treatments for PCB disposal, the use of microbial resources for their degradation/transformation has been extensively studied and accepted as an eco-friendly and low-cost alternative. Various microorganisms belonging to diverse taxonomic groups (bacteria, cyanobacteria, and fungi) have been recognized as catabolically active in degrading/transforming PCBs (Xiang et al., 2020), including different bacterial genera

Pseudomonas, *Burkholderia*, *Comamonas*, *Cupriavidus*, *Sphingomonas*, *Acidovorax*, *Rhodococcus*, *Ralstonia*, *Bacillus*, cyanobacteria *Anabaena* or white rot fungi *Phanerochaete* (Abraham, 2002.; Bedard, 1986; Cvančarová et al., 2012; Garrido-Sanz et al., 2018; Komancova et al., 2003; Pieper, 2005; Sakai et al., 2002; Seto et al., 1995; Sharma et al., 2018; Zhang et al., 2015, Wang et al., 2018). Each of these microbial isolates shows quite unique spectra in regard to the type and extent of PCB congeners that they metabolize, with most, unfortunately, having rather narrow specificity, allowing the transformation of only a small number of PCBs (Arnett et al., 2000; Borja et al., 2005, McKay et al., 2003; Mondello et al., 1997). The genetic background for the observed PCB-degrading ability has been studied in many of these strains (Erickson et al. 1993; Hofer et al., 1994; Pieper, 2005), however, most of our knowledge on the PCBs biochemistry, metabolism and organization of catabolic genes still comes from model bacterial strains, *Paraburkholderia xenovorans* LB400, *Pseudomonas pseudoalcaligenes* KF707 and *Rhodococcus jostii* RHA1 whose genomes have been fully sequenced (Chain et al., 2006; Furukawa et al., 2008; McLeod et al., 2006)

The biphenyl (*bph*) catabolic pathway, presented in **Fig. 1,** has been identified as a main pathway through which PCBs are aerobically transformed by bacteria (Furukawa et al., 2004, Pieper, 2005). The pathway is initiated by the attack of biphenyl dioxygenase (BphA) on a 2,3(5,6) position of the, preferentially non-substituted or less substituted, biphenyl ring. The formed dihydrodiol is transformed in the second step by 2,3-dihydrodiol-2,3-dehydrogenase (BphB) to 2,3-dihydroxybiphenyl which is further cleaved by the enzyme 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) to yield a *meta*-cleavage product 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA). The last step, catalyzed by 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (BphD), generates two final metabolites of the upper *bph* pathway: benzoic acid and 2-hydroxypenta-2,4-dienoate. In addition to this pathway, it has

been found that certain bacteria can transform congeners that occupy the 2,3(5,6) positions of the ring, by oxygenation proceeding at 3,4 positions, with the proposed formation of acetophenones as the final metabolites (Bedard et al., 1990; Erickson et al., 1993; Gibson et al., 1993; Komancova et al., 2003). A complete and well-rounded understanding of this second PCB transformation pathway remains to be reached; however, PCB-pioneer researchers (Ahmad et al., 1991) have suggested several acidic metabolites found in culture extracts as intermediary metabolites leading to acetophenone formation.

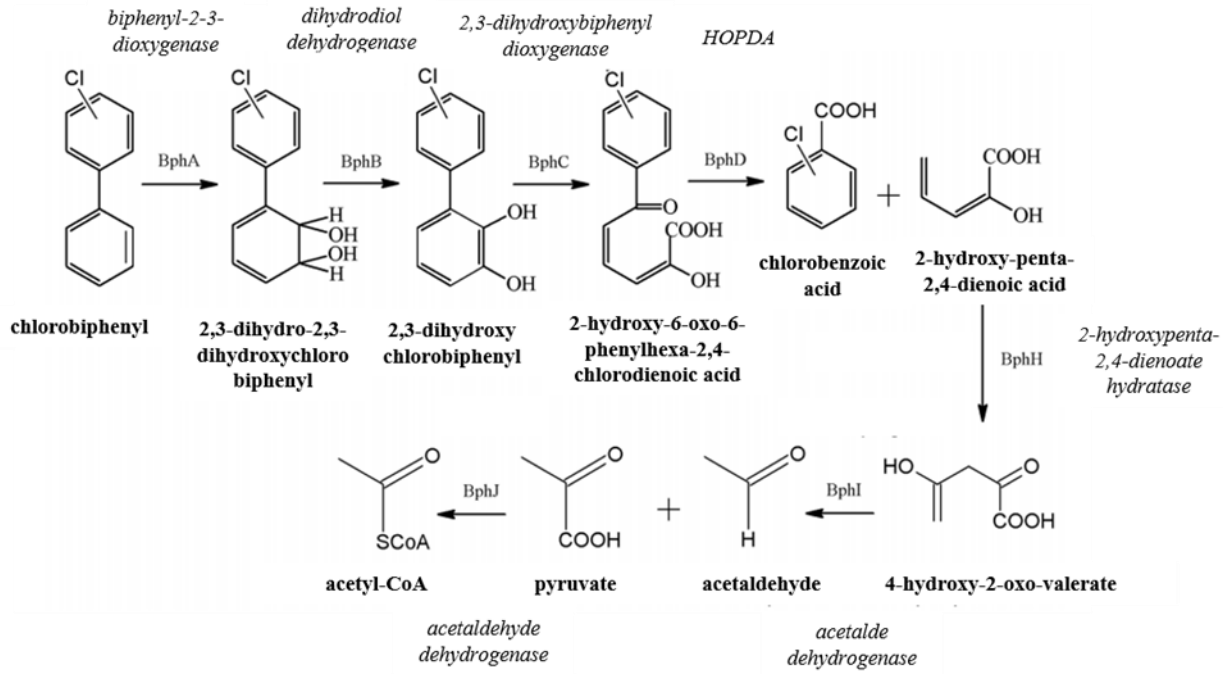


Fig. 1. The principal steps in *bph pathway* involved in PCBs microbial transformation. BphA (biphenyl-2,3-dioxygenase) catalyzes the transformation of biphenyl (1) to biphenyl-2,3-dihydrodiol (2). BphB (biphenyl-2,3-dihydrodiol-2,3-dehydrogenase) forms 2,3-dihydroxybiphenyl (3), BphC (2,3-dihydroxybiphenyl-1,2-dioxygenase) 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (4), and BphD (2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase) 2-hydroxypenta-2,4-dienoate (5a) and benzoic acid (5b).

It is clear that our knowledge on enzymes and pathways involved in the transformation of PCBs and its metabolites is still rather fragmentary given the pleiotropic strategies of microorganisms to transform PCBs. Already in 2008, Pieper and Seeger stated that the PCB metabolism should not be regarded as a simple linear pathway, but as a complex interplay

between different catabolic pathway modules. As shown, depending on the congener itself, and the specificity of the Bph enzymes harboured by a given organism, different *bph* pathway steps may constitute metabolic bottlenecks, resulting in the accumulation of the respective toxic metabolites which can be viewed as a kind of suicide inactivation of different Bph pathway enzymes (Dai et al., 2002; Garrido-Sanz et al., 2018). Bacterial strains using different pathways interplay, offering them a way to by-pass these issues, would have greater PCB-degrading ability. One such bacteria could be those belonging to the genus *Rhodococcus*, known for its high metabolic versatility in degrading different xenobiotics and by harbouring multiple and unique enzyme systems (Garrido-Sanz et al., 2020, Iwasaki et al., 2006; Masai et al. 1995; Seto et al. 1995; Taguchi et al., 2004; Van der Geize, 2004; Yang et al., 2004). During our previous studies on PCB-contaminated soils and marine sediments, we have successfully isolated several *Rhodococcu*s strains capable of transforming a wide range of structurally different PCB congeners (Begonja Kolar et al., 2007, Petrić et al., 2007), a feature that could not be explained if only the known *bph* pathway was considered. Within the genome of several PCB-degrading rhodococci, *bph* genes encoding multiple pathway isoenzymes have already been identified (Pieper, 2005; Shimizu et al., 2001). Even so, information on the potential multiple pathway and complete array of intermediate metabolites produced during PCB transformation by rhodococci is still unavailable.

To obtain better insight into the PCB transformation mechanisms used by rhodococci, we designed an experiment in which we used gas chromatographic – mass spectrometric (GC-MS) analysis to monitor and identify transformation products (TPs) formed during the transformation of 3 selected (structurally) different PCB congeners: 2,4,4'-trichlorobiphenyl (PCB-28), 2,2',5,5'-tetrachlorobiphenyl (PCB-52) and 2,4,3'-trichlorobiphenyl (PCB-25). The potential wider spreading of the observed mechanisms within the *Rhodococcus* genus was studied by including four rhodococcal strains into the experiment, namely Z6, T6, R2, and

Z57, isolated from different environments (Begonja Kolar et al., 2007, Petrić et al., 2007). Under the presumption that the full catabolic potential of rhodococci in PCB biodegradation is yet to be discovered, we aimed to determine the level of specificity of the enzymes involved in PCB biotransformation, having in mind the potential of our study to unravel an existence of multiple PCB-degrading pathways in rhodococci.

2. Materials and methods

2.1. Bacterial strains, culture media and chemicals used

The PCB-degrading bacterial strains used in the study included *Rhodococcus* isolates Z6, T6, R2, and Z57, with details on each isolate provided in **Table 1** (Begonja Kolar et al., 2007, Petrić et al., 2007). As a basal medium, we used phosphate-buffered minimal salts medium (PAS), both for growth of the strains and for biodegradation experiments (Bedard et al., 1986). Its modified version was used for the strains originating from marine sediments (MPAS) by mixing filter sterilized seawater and PAS medium at 1:1 (Begonja Kolar et al., 2007). The PCB congeners, 2,4,4'-trichlorobiphenyl (2,4,4'-CB), 2,2',5,5'-tetrachlorobiphenyl (2,2',5,5'-CB) and 2,4,3'-trichlorobiphenyl (2,4,3'-CB), used in the study were purchased from Sigma-Aldrich as BCR® certified Reference Materials. Stock solutions of individual congeners were prepared in acetone (1mg/ml). Biphenyl ($C_6H_5C_6H_5$; 99.5 % purity) and other high purity chemicals used for the extraction of metabolites and GC-MS analysis were purchased from Sigma-Aldrich and Merck (Darmstadt, Germany).

152 **Table 1.** Information on the PCB degrading *Rhodococcus* strains used in the study.

Label	16S rRNA gene identity	Enriched from the mixed culture	Mixed culture origin [PCB mass fraction in sample (mg kg^{-1})][a]	Sampling location
Z6	*Rhodococcus* sp.[b]	TSZ7	Transformer station soil [6044]	Zadar, Croatia
T6	*Rhodococcus* sp.[b]	AIR1	Airport soil [2.210)	Trogir, Croatia
R2	*Rhodococcus erythropolis*[c]	RMC2	Harbour marine sediment [0.495]	Rijeka, Croatia
Z57	*Rhodococcus ruber*[c]	ZMC57	Harbour marine sediment [0.540]	Zadar, Croatia

153 [a] PCB mass fraction in sample (mg kg^{-1}) determined against Aroclor 1248+1254 standards
154 [b] Petrić et al., 2007
155 [c] Begonja Kolar et al., 2007

156

157 ***2.2. PCB biodegradation assays***

158 Biodegradation assays were performed in 50-mL Erlenmeyer flasks containing (i) 20 ml
159 of sterile PAS or MPAS medium, (ii) the selected individual PCB congener, provided at a
160 final concentration of 5 mg l^{-1}, (iii) the biphenyl, used as an inducer, provided at a final
161 concentration of 250 mg l^{-1}, and (iv) 2 ml of individual bacterial inoculum. Starter bacterial
162 inoculums (3-day old cultures) were prepared for the experiment by growing selected
163 *Rhodococcus* strains in PAS or MPAS medium supplemented only with biphenyl at a
164 concentration of 1 g l^{-1}. Flasks without bacterial inoculums were likewise included in the
165 assay, representing controls. These controls allowed us to follow the potential abiotic
166 transformation of the selected PCB congeners. The prepared flasks were incubated on a
167 rotary shaker at 200 rpm and 28 ^{o}C, under aerobic conditions (flasks were plugged with
168 cotton pads). Biodegradation assay was conducted over seven days. All assays were
169 performed in duplicates.

The PCB transformation potential of strains Z6, T6, R2 and Z57 was investigated using two model congeners: (i) 2,4,4'-CB, with all 3,4 ring positions blocked, chosen as a model to follow the attack of BphA on the free 2,3 position, and (ii) 2,2',5,5'-CB, with all 2,3 ring positions blocked, chosen as a model to follow the attack of BphA on a 3,4 position. In addition, the potential of strains R2 and Z57 to transform congener 2,4,3'-CB was also investigated, both having free positions for a 2,3 and 3,4 attack.

2.3. Gas chromatographic- mass spectrometric analysis

To assure an accurate chemical analysis of compounds (PCBs, TPs), we decided to process the whole volume of the sample and therefore samples were taken for the GC-MS analysis only at the end of the experiment (at day 7). This was required due to the hydrophobic properties of PCBs, e.g. potential sorption. Hence, whole flask volumes were submitted to the extraction procedure described previously in detail (Petrić et al., 2007). Briefly, two organic layers obtained from neutral extraction (with dichloromethane) and acid extraction (with dichloromethane and hydrochloric acid) were joined. After drying over anhydrous sodium sulfate and concentration by a gentle stream of nitrogen, extracts were subjected to GC-MS using a GC/MS HP Agilent instrument (Palo Alto,CA, USA). The instrument was equipped with a DB-5MSITD column (30 m x 0.25 mm, film thickness 0.25 μm) (J&W Scientific, Folsom, USA) with helium used as a carrier gas. The column was heated from 35 ^{o}C (1min hold) to 300 ^{o}C at 5 ^{o}C min^{-1} rate (15 min hold). Mass spectra of the transformation products were recorded in the full scan acquisition mode (mass range 33-650 *m*/*z*) and the scan rate of 1 scan s^{-1}. The injection volume of the sample was 2 μl. Given that the vast majority of the TPs did not exist within database libraries (NIST, Wiley), their identification was performed by a comprehensive examination of the fragmentation patterns of the mass spectra ions.

3. Results

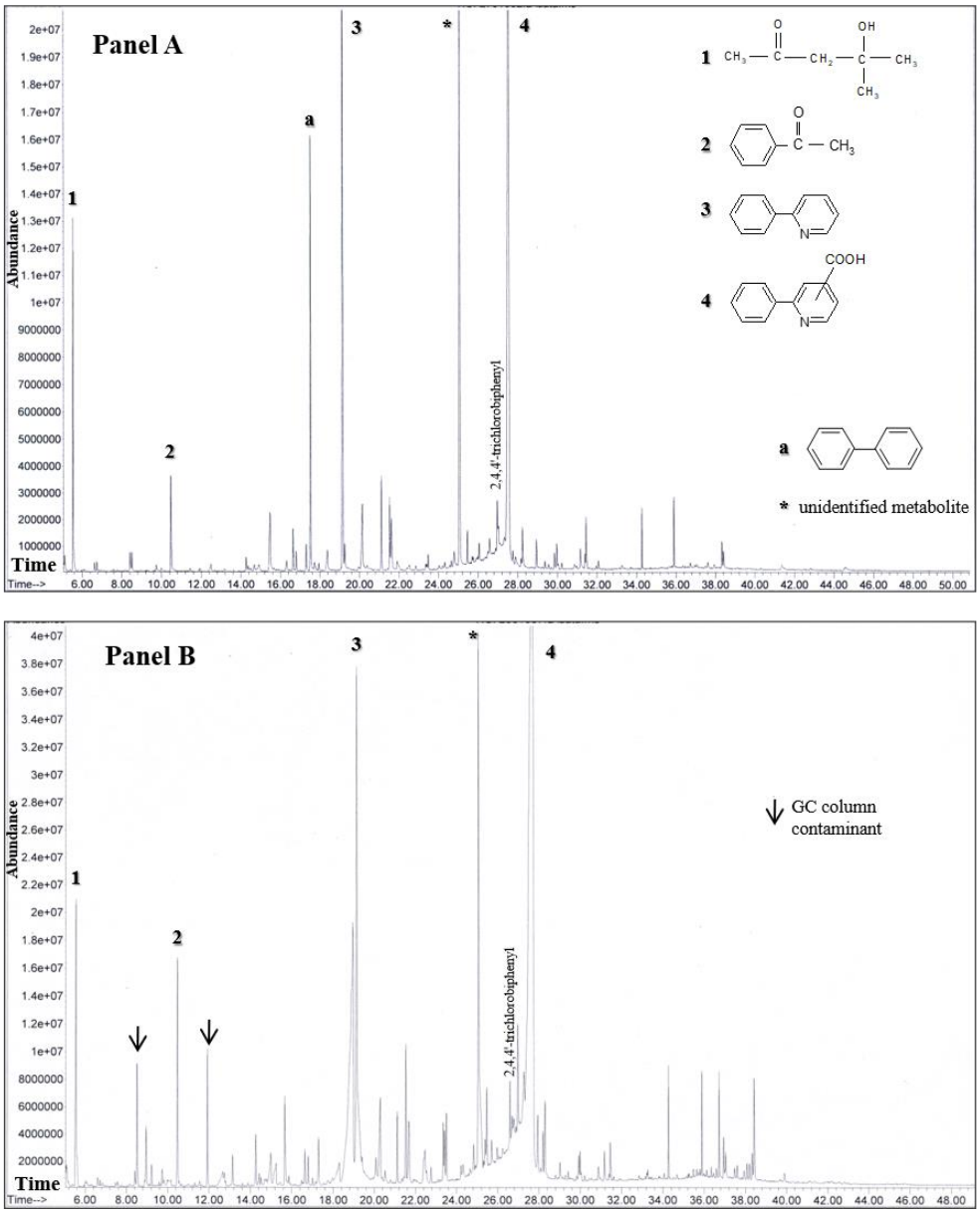
A visual examination of the flasks at the end of the 7-day biodegradation experiment suggested that all 4 tested rhodococci successfully grew in the presence of a biphenyl (observed turbidity), while an intensive yellow color (indication of the *meta*-cleavage product HOPDA) suggested biotransformation of biphenyl and/or model congeners. Biotic transformation of the compounds was further confirmed by (i) visual examination of the control flasks in which no changes in the turbidity and/or color were observed and (ii) GC-MS analysis of the control flasks in which both the originally added biphenyl and individual PCB congener were detected in the extracts. In addition, we detected one biphenyl transformation product (with an additional methyl group on one phenyl ring) in the controls, which was presumed to represent an impurity of the biphenyl used in the experiment.

3.1. Transformation of 2,4,4'-CB by the selected rhodococci

Total ion current GC-MS chromatograms of the culture extracts obtained for all rhodococcal strains at the end of the experiment with 2,4,4'-CB are presented in **Figs. 2A** and **2B**, while the list and presumed formulas of the identified TPs are given in **Tables 2A** and **2B.** In addition, the mass spectra of some of the key TPs detected in this assay are provided in the Supplementary material (**Fig S1**). Residual amounts of the originally added biphenyl and 2,4,4'-CB congener (peak with symbol "a" and peak "2,4,4'-trichlorobiphenyl", **Fig. 2A**) indicated that the transformation process was still not finished at day 7. Still, the formation of a number of TPs was commonly observed in extracts of all rhodococcal isolates. They included both non-chlorinated biphenyl TPs (**Table 2A**), but likewise an array (up to 19 depending on the strain) of chlorinated TPs carrying a phenyl ring as a molecule substituent (**Table 2B**). From an array of different chlorinated TPs, tagged as those deriving from 2,4,4'-CB, only 4 TPs were shared in all 4 tested rhodococci and included dichlorinated benzoic

218 acid (CB2) and acetophenone (CB3), 2,4-dichlorophenylacetic acid (CB7) and 3-(2,4-
219 dichlorophenyl)-2-propenoic acid (dichlorinated cinnamic acid) (CB10). Other TPs included
220 an array of different phenyl compounds with an aliphatic side chain containing from 1 to 5 C
221 atoms: (i) carboxylic acids R–COOH (such as phenylpropenoic (cinnamic) and propanoic
222 acids or phenyl acetic acids) or (ii) alcohols R-OH (such as phenol, phenyl methanols or
223 phenyl ethanoles).

224 Interestingly, apart from the expected TPs carrying a phenyl ring as a substituent, an
225 array of additional TPs was identified in the analyzed extracts. One was a linear metabolite,
226 identified as 4-hydroxy-4-methyl-2-pentanone (peak no. 1, **Figs. 2A** and **2B**) detected in all
227 of the culture extracts. In addition, unexpected TPs included those containing pyridine
228 moieties as well as TPs built up of up to three phenyl rings (**Fig. 3**), identified in all culture
229 extracts (such as 2-phenyl pyridine, 2-phenyl-3-hydroxypyridine, 2-phenylpyridine
230 carboxylic acid, 2-(2,4-dichlorophenyl) pyridine, 2-(2,4-dichlorophenyl)-5-chloropyridine,
231 2,4-dichlorophenyl-4-chlorophenyl-ketone, di-(2,4-dichlorophenyl)-ketone). Many of these
232 unexpected peaks also represented the most prominent TPs standing out from the others in
233 GC-MS chromatograms by their peak intensities (peak heights) (peak nos. 1, 3, 4, 6, 7 and 9,
234 **Figs. 2A and 2B**). High intensity TP, eluting from the column around minute 25, was not
235 positively identified (peak with an asterisk symbol, **Figs. 2A** and **2B**). In the assays with
236 strains Z6, T6 and R2, one of these prominent TPs corresponded to non-chlorinated
237 acetophenone (peak no. 2, **Figs. 2A** and **2B**). Compared to strains Z6 and T6, chromatograms
238 of strains R2 and Z57 included greater numbers of high intensity peaks. Unfortunately, with
239 no standards available, it was not possible to determine the actual concentration of each of the
240 detected TPs.



241

242 **Fig. 2A.** Full-scan GC-MS chromatograms of the culture extracts after 7-day of incubation of
243 Rhodococcus sp. Z6 (panel A) and Rhodococcus sp. T6 (panel B) with 2,4,4'-trichlorobiphenyl in the
244 presence of biphenyl.

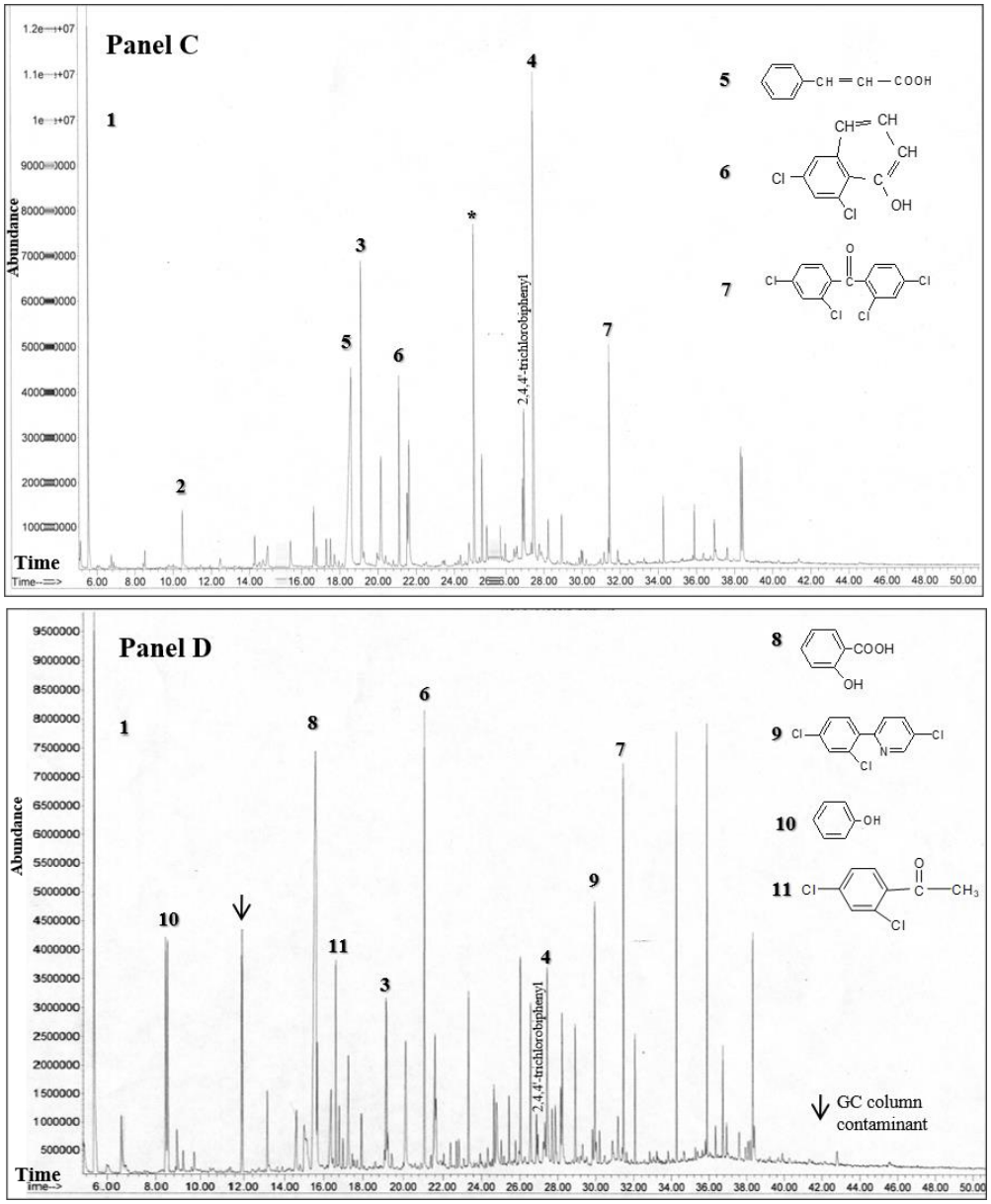
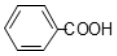
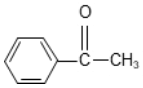
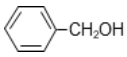
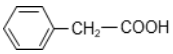
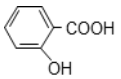
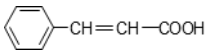
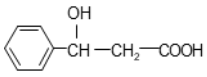
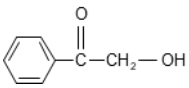


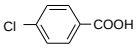
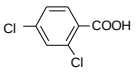
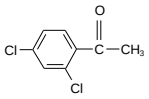
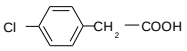
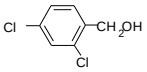
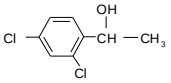
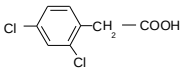
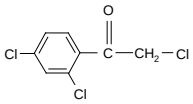
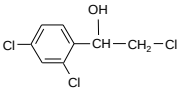
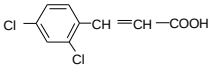
Fig. 2B. Full-scan GC-MS chromatograms of the culture extracts after 7-day incubation of *Rhodococcus erythropolis* R2 (Panel C) and *Rhodococcus ruber Z57* (Panel D) with 2,4,4'-trichlorobiphenyl in the presence of biphenyl

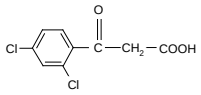
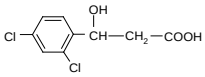
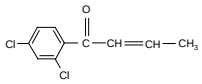
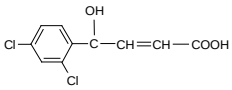
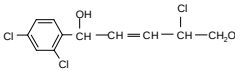
Table 2A. Identification and mass spectra of the TPs formed from biphenyl during 2,4,4'-trichlorobiphenyl biodegradation in cultures of *Rhodococcus* Z6, T6, R2 or Z57.

Transformation product (TP) designation	TP	TP formula	TP *m/z*[a]	Microbial culture
B1	Benzoic acid		122,105,77,51	Z6, T6, R2
B2	Acetophenone		120,105,77,51	Z6, T6, R2
B3	Phenol		94,66,50	all
B4	Benzyl alcohol		108,91,79,51	all
B5	2-phenylacetic acid		39,65,91,136	Z6, Z57
B6	Salicylic acid		64,92,120,138	Z6, R2, Z57
B7	Cinnamic acid		147,130,103,77	Z6, T6, R2
B8	3-hydroxy-3-phenylpropanoic acid		166,107,79,51	Z6, T6, R2
B9	2-hydroxy-1-phenyl-ethanone		51,77,105,136	Z6

[a] Main mass spectra *m/z* ions

Table 2B. Identification and mass spectra of the chlorinated TPs formed from 2,4,4'-trichlorobiphenyl during biodegradation in cultures of Rhodococcus Z6, T6, R2 and Z57.

Transformation product (TP) designation	TP	TP formula	TP *m/z*[a]	Microbial culture
CB1	**4-chlorobenzoic acid**		50,75,111,139,156	Z6, Z57
CB2	**2,4-dichlorobenzoic acid**		74,109,145,173,190	all
CB3	**2,4-dichloroacetophenone**		75,109,145,173	all
CB4 2C[b]	4-chlorophenylacetic acid		44,92,170,125,44	Z57
CB5 2C[b]	2,4-dichlorophenyl methanol		51,77,111,141,176	Z6, R2
CB6 2C[b]	α-methyl-2,4-dichlorophenyl methanol		43, 75,111,147,175	Z6, Z57
CB7 2C[b]	2,4-dichlorophenylacetic acid		63,89,125,159,204	all
CB8 2C[b]	2-chloro-1-(2,4-dichlorophenyl)ethanone		75,109,145,173	Z6, T6
CB9 2C[b]	2,4-dichloro-α-(chloromethyl)benzenemethanol		50,75,111,147,175	Z6, R2
CB10 3C[b]	3-(2,4-dichlorophenyl)-2-propenoic acid		74,99,125,136,181,216	all

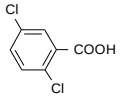
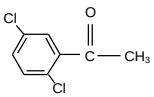
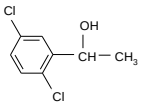
CB11 3C[b]	3-(2,4-dichlorophenyl)-3-oxo-propanoic acid		232,216,173,145	R2, Z57
CB12 3C[b]	1-hydroxy-1-(2,4-dichlorophenyl)-2-propanoic acid		232,214,198,175	R2
CB13 4C[b]	1-(2,4-dichlorophenyl)2-butene-1-on		111, 173,145	Z6, T6
CB14 4C[b]	4-(2,4-dichlorophenyl)-4-hydroxy-2-butenoic acid		230,195,175,56	Z57
CB15 5C[b]	5-(2,4-dichlorophenyl)-5-hydroxy-2-chloropentenol		282,252,188,175	R2, Z57

[a] Main mass spectra *m/z* ions
[b] Number of C in the aliphatic side chain

3.2. Transformation of 2,2',5,5'-CB by the selected rhodococci

In comparison with the previously studied congener, a much smaller number of peaks was detected in GC-MS chromatograms of the 2,2',5,5'-CB biodegradation media at the end of the experiment (**Fig S2**), with strain Z57 characterized by the smallest number of detected TPs. Some of the TPs were identical to those found in the 2,4,4'-CB assay and included non-chlorinated compounds formed from biphenyl: (i) B1 (benzoic acid), (ii) B2 (acetophenone), (iii) B4 (benzyl alcohol) and (iv) B7 (cinnamic acid), identified in Z6, T6 and R2 assays, and in addition (v) B6 (salicylic acid), identified in the assay of strain R2 (**Table 2A**). From chlorinated TPs, carrying a phenyl ring as a molecule substituent, we identified only 3 compounds (**Table 3**): (i) dichlorinated benzoic acid (CB16), (ii) dichloroacetophenone (CB17) and (iii) α-methyl-2,5-dichlorobenzyl alcohol (CB18). Once more, in all culture extracts, an unexpected linear TP (4-hydroxy-4-methyl-2-pentanone) and TPs containing pyridine moieties (such as 2-phenylpyridine and/or 2-phenylpyridine-carboxylic acid) were identified (**Fig S2**). High residual amounts (high peaks) of the originally added biphenyl and 2,2',5,5'-CB were detected at the end of the experiment (**Fig S2**). Higher prevalence of the following, above mentioned TPs, was recorded in the culture extracts: 4-hydroxy-4-methyl-2-pentanone, non-chlorinated acetophenone (B2), cinnamic acid (B7) and 2,5-dichlorobenzoic acid (CB16) (**Fig S2**).

277 **Table 3.** Identification and mass spectra of chlorinated TPs formed from 2,2',5,5'-tetrachlorobiphenyl
278 during biodegradation in cultures of Rhodococcus Z6, T6, R2 and Z57. ***** Metabolites marked as B1,
279 B2, B4 and B7 presented in the Table 2 were also identified in culture extracts of isolates Z6, T6 and
280 R2. In addition metabolite B6 was identified in the R2 isolate culture extracts.

Transformation product (TP) designation	TP	TP formula	TP *m/z*[a]	Microbial culture
CB16	**2,5-dichlorobenzoic acid**		74,109,145,173,190	Z6, T6, R2
CB17	**2,5-dichloroacetophenone**		71,145,175,188	all
CB18 2C[b]	α-methyl-2,5-dichlorobenzyl alcohol		111,147,175,190	all

281 [a] Main mass spectra *m/z* ions
282 [b] Number of C in the aliphatic side chain
283

284 ### *3.3. Transformation of 2,4,3'-CB by the selected rhodococci*

285 The transformation activity towards congener 2,4,3'-CB was tested only on two of the
286 selected isolates, Z57 and R2 (**Fig S3**) with an identified small number of non-chlorinated
287 and chlorinated TPs, mostly analogous to those detected in the biodegradation assays with
288 either 2,4,4'-CB or 2,2',5,5'-CB congener. We found relatively high residual amounts of the
289 originally added congener 2,4,3'-CB in both culture extracts. The TPs identified in the culture
290 extract included non-chlorinated TPs (i) B2 (acetophenone) and (ii) B8 (3-hydroxy-3-
291 phenylpropanoic acid), (iii) dichlorinated acetophenone (CB3), (iv) α-methyl-2,4-
292 dichlorophenyl methanol (CB6) and (v) 2-phenylpyridine, with the formula already presented

293 in **Tables 2A** and **2B** and in **Fig. 3**. The three last TPs represented peaks with the highest
294 intensity in the GC-MS chromatograms (**Fig S3**). When compared to R2, a new TP - 2,4-
295 dichlorobenzaldehyde (*m/z* 75,111,145,173; in 11.45 min) was identified in the culture
296 extracts of the isolate Z57.

297 **4. Discussion**

298 The PCB metabolism in rhodococci is presumed to be a complex interplay between
299 different catabolic pathway modules, however, clear information on these pathways still
300 remains to be attained. In our study, we focused on identifying all of the TPs produced during
301 the biotransformation of PCBs by 4 studied rhodococcal strains, and based on the key TPs
302 detected (mainly in the assay with 2,4,4'-CB), we proposed tentative pathways used by
303 rhodococci for PCB transformation (**Fig. 4**). As explained in more details in the following
304 paragraph of the discussion, these pathways did not rely merely on the regular *bph* pathway
305 leading to chlorobenzoic acids formation (**Fig. 4,** Scheme upper part, step 1a and 1b,
306 Paragraph 4.1), but also included additional pathways: benzoic acids formation (i) via
307 stepwise oxidative decarboxylation of the aliphatic side chain (**Fig. 4,** Scheme upper part,
308 step 2, explained in Paragraph 4.4.) and (ii) via intradiol opening of the biphenyl ring (**Fig. 4,**
309 Scheme upper part, step 3, explained in Paragraph 4.4.**)** and also pathway leading to
310 acetophenone formation (**Fig. 4,** Scheme lower part, explained in Paragraph 4.3.). The
311 existence of multiple pathways in single strains has up until now been proposed only in
312 *Rhodococcus jostii* RHA1 (Iwasaki et al., 2006) and *Rhodococcus* sp. WAY2 (Garrido-Sanz
313 et al., 2018).

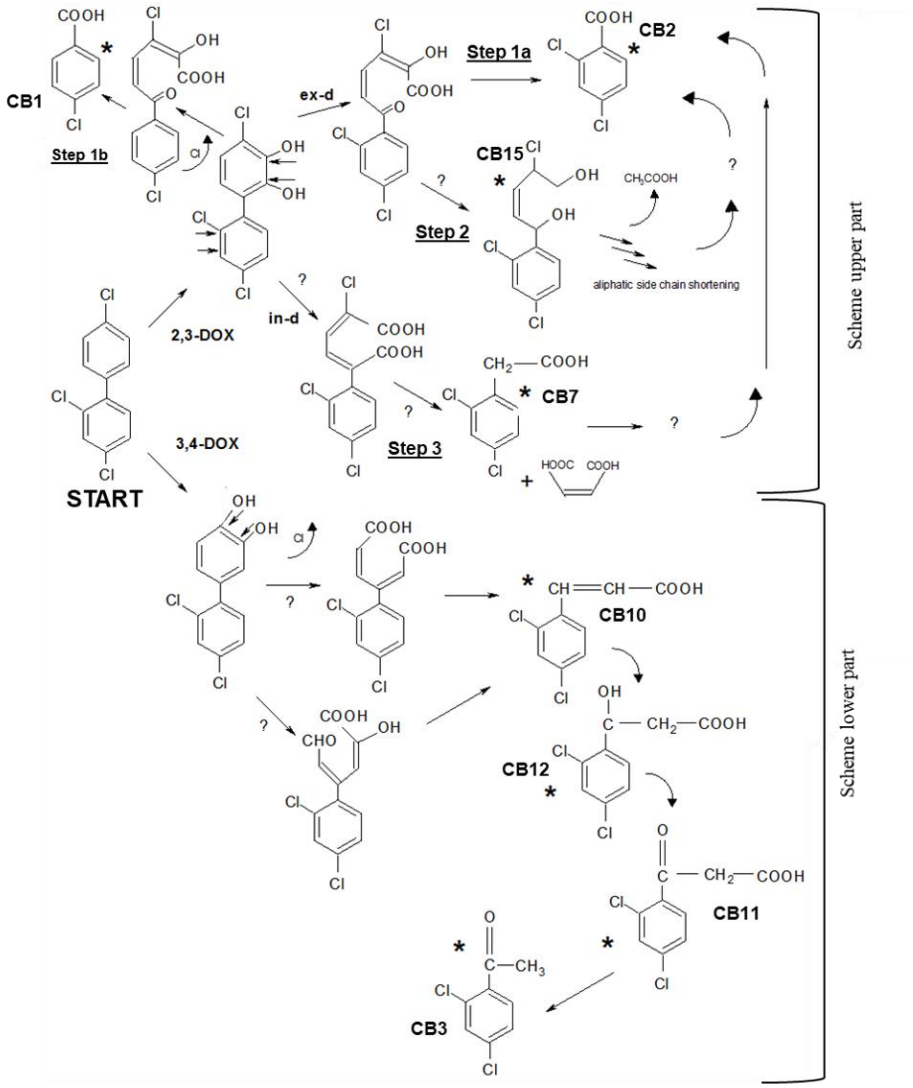


Fig. 4. Tentative pathways proposed to be active in rhodococci during biotransformation of congener 2,4,4`-CB (START):

(1) 2,3-dioxygenation (2,3-DOX) of the biphenyl ring (upper part of the scheme)

- *regular bph pathway* leading to the production of 2,4-dichlorobenzoic acid (CB2) **(Step 1a)** and 4-chlorobenzoic acid (CB1) (**Step 1b).**
- *alternative patway* working *via* shortening of the HOPDA aliphatic side chain (**Step 2**) with 5–(2,4-dichlorophenyl)-5-hydroxy-2-chloropentenol (CB15) as one of the TP, leading to the production of 2,4-dichlorobenzoic acid (CB2).
- *alternative pathway* working via intradiol opening of the biphenyl ring (**Step 3**) with 2,4-dichlororphenylacetic acid (CB7) as one of the TP, leading to the production of 2,4-dichlorobenzoic acid (CB2).

(2) 3,4-dioxygenation (3,4-DOX) of the biphenyl ring (lower part of the scheme)

- *alternative pathway* leading to the production of 2,4-dichlororacetophenone (CB3) with dichlorinated cinnamic acid (CB10), 3-(2,4-dichlorophenyl)-3-oxo-propanoic acid (CB11) and 1-hydroxy-1-(2,4-dichlorophenyl)-2-propanoic acid (CB12) as TPs.

* TPs identifed in the assay with 2,4,4`-CB with details provided in Table 2B
in-d = intradiol opening
ex-d = extradiol opening

334 ***a. Existence of regular pathway leading to chlorobenzoic acids formation***

335 Identification of chlorinated benzoic acids in the culture extracts confirmed that our
336 rhodococci followed a *bph* pathway initiated by the primary dioxygenase attack on the
337 2,3(5,6) positions of the biphenyl ring. Although the less chlorinated ring was shown to be
338 more susceptible to bacterial transformation, strains Z6 and Z57 showed additional ability to
339 attack both rings of the 2,4,4'-CB congener, as evident from the detection of both di- and
340 mono-chlorinated benzoic acids (**Fig. 4,** step 1a and 1b). In addition, the unexpected
341 detection of chlorinated benzoic acids also in the assay with congener 2,2',5,5'-CB, with no
342 required free 2,3(5,6) positions for the initial enzyme attack, indicated very flexible and
343 unusual dioxygenase harboured by our rhodococcal strains. Based on the available literature
344 we propose two hypotheses for the observed benzoic acids formation in assay with 2,2',5,5'-
345 CB: (i) dechlorination of one of the phenyl rings at -*ortho* position could have preceded 2,3-
346 dihydroxylation, after which this congener followed the regular *bph* pathway or (ii) benzoic
347 acids were formed after an initial attack at free 3,4(5,6) positions following a non-regular
348 pathway - *via* the production of chlorinated cinnamic acid as an intermediate TP. This
349 seldomly reported pathway (Ahmad et al., 1991; Hilton and Cain 1990) proposes a chain of
350 reaction that includes the transformation of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (or
351 its saturated analogues) to chlorinated cinnamic acid and then to chlorinated benzoic acid.
352 Chlorinated cinnamic acids, even though detected in the assay with 2,4,4'-CB, were not
353 identified in the assay with 2,2',5,5'-CB congener, therefore preventing us from reaching a
354 firm final conclusion on one of these hypotheses. Of the four tested rhodococci, only strain
355 Z57 was not shown to possess this transformation ability.

356 Chlorobenzoic acids, formed at the end of the known upper *bph* pathway, are generally
357 further transformed very slowly by PCB-degrading bacteria, making this step a rate limiting

stage in the overall PCB-biodegradation (Zhang et al., 2009). Based on some of the identified TPs (e.g. phenols, benzyl alcohols and 2,4-dichlorophenylmethanol), we propose that benzoic acids are not necessary dead-end intermediates in PCB transformation but are further transformed *via* initial 1,2-dioxygenation (Davison et al., 1999). The latter two of these TPs have been previously associated with the transformation of chlorinated benzoic acids by ligninolytic fungi (Muzikár et al., 2011).

b. The existence of an alternative pathway leading to acetophenones formation

Specificity of dioxygenase harboured by rhodococcal strains

The identification of chlorinated acetophenones in the culture extracts suggested that an alternative pathway was also active in our rhodococcal isolates. As proposed by other authors, if the chlorine atoms are blocking the 2,3(5,6) positions, oxidation of the biphenyl ring occurs *via* a 3,4(4,5) attack, with acetophenones arising as the final TPs (Bedard et al., 1990; Erickson et al., 1993; Gibson et al., 1993; Komancova et al., 2003). Following this scheme we expected to detect acetophenones only in assays with congeners 2,2',5,5'-CB and 2,4,3'-CB; however, we likewise found them to be formed from 2,4,4'-CB. This indicated the specific nature of dioxygenase harboured by all four rhodococci able (i) to attack both 2,3(5,6) and 3,4(4,5) positions of the biphenyl rings but likewise (ii) to dechlorinate PCB molecules. The studied rhodococcal strains might either harbor very specific Biphenyl dioxygenase or the observed activity could be due to a combined action of different ring-hydroxylating dioxygenase as part of the *etb* (ethylbenzene) *ben* (benzoate), *cat* (catechol), *pca* (protocatechuate) or *nar* (naphthalene) pathways, known to have overlapping functions (Garrido-Sanz et al., 2018; Iwasaki et al., 2006; Kimura et al., 2006; Kitagawa et al., 2001a and 2001b; Sakai et al., 2003).

The mechanisms previously proposed (Bedard et al., 1990; Haddock et al., 1995,) indicate that ring-hydroxylating dioxygenases might concurrently also conduct dechlorination. Based on the chlorine substitution patterns of the congeners used in the assays, we propose that our rhodococci holds the dechlorination ability for the -*para* and -*ortho* position of the biphenyl ring. PCB-degraders usually do not have the ability to also dehalogenate PCBs, but several successful strains, including some rhodococci, can be found throughout the literature (Ahmad et al., 1991 ; Haddock et al., 1995 ; Komancovà et al., 2003 ; Rybkina et al., 2003; Pieper, 2005 ; Yang et al., 2004). The proposed dechlorination ability further supports the first hypothesis postulated earlier about the formation of chlorinated benzoic acids in the assay with congener 2,2',5,5'-CB.

Interestingly, the identification of non-chlorinated acetophenone, presumed to be formed from biphenyl, further implied that the proposed alternative pathway is not activated in the case of blocked 2,3(5,6) positions, but rather acts as a parallel pathway. The specificity of rhodococcal dioxygenases were further revealed in the assay with a third model congener 2,4,3'-CB. The preferential detection of dichlorinated acetophenone over benzoic acid indicated that our rhodococci might follow an alternative pathway for PCB transformation rather than the regular one.

c. The proposed pathway leading to acetophenone formation

Identification of certain "acidic" TPs in the culture extracts and the available, but scarce literature reports (Hilton and Cain, 1990), allowed us to propose part of the alternative pathway leading to the formation of the identified acetophenones (**Fig 4,** lower part of the Scheme). We propose that chlorinated "acidic" TPs [dichlorinated cinnamic acid – CB10, 3-(2,4-dichlorophenyl)-3-oxo-propanoic acid – CB11 and 1-hydroxy-1-(2,4-dichlorophenyl)-2-propanoic acid – CB12] take part in the alternative pathway, all preceding the formation of

acetophenone. Unfortunately, due to the small number of TPs identified in the assays with congeners 2,2',5,5'-CB and 2,4,3'-CB, these conclusions are only based on the data obtained with congener 2,4,4'-CB. However, it was evident that the formation of these unexpected "acidic" TPs, obviously not part of the regular *bph* pathway, was not limited to PCB transformation but was also used simultaneously by our four rhodococci for biphenyl transformation (detection of non-chlorinated "acidic" TPs: cinnamic acid and 3-hydroxy-3-phenylpropanoic acid). Finally, the high intensities of peaks corresponding to TPs such as acetophenones (non- and di-chlorinated) and cinnamic acid implicated the importance of this alternative pathway in PCB transformation by our rhodococcal strains.

d. The existence of an additional transformation pathway for chlorobenzoic acid formation

Additionally, the spectrum of the identified phenylic compounds carrying an aliphatic side chain containing two to five carbon atoms (different carboxylic acids R–COOH and alcohols R-OH) that could not be attributed to neither regular *bph* pathway nor the proposed alternative pathway, indicated the existence of even more pathway(s) for PCB transformation by the studied rhodococci. These types of compounds can scarcely be discovered in the literature and have up until now been detected only in several PCB-biodegradation assays conducted with *Pseudomonas, Achromobacter* and *Bacillus* (Ahmad et al. 1991; Masse et al., 1989). Based on this study, we propose that these phenylic compounds carrying an aliphatic side chain are not dead-end intermediates generated by the spontaneous cleavage of some unstable intermediate, but are generated by the *meta*-cleavage of the HOPDA, formed by the regular *bph* pathway. We propose that this third pathway involved stepwise oxidative decarboxylation of the aliphatic side chain of the HOPDA (**Fig. 4**, step 2) leading finally to alternative way of chlorobenzoic acid formation**.** As similarly concluded by Ahmed et al.

(1991), some bacterial strain do not necessarily convert HOPDA directly to benzoic acid but *via* its saturated analogues. Curiously, the identification of one specific TP, namely 2,4-dichlorophenylacetic acid (CB7, **Fig. 4**), allowed us to speculate about the possibility of a fourth additional pathway leading to the formation of chlorobenzoic acids, which occurred *via* an intradiol opening of the biphenyl ring (**Fig. 4**, step 3).

This experiment, unfortunately, did not allow us to define how this pathway is regulated i.e. if an alternative pathway functions in parallel from the beginning of the transformation process or is activated at a later step during the transformation. Studies have clearly shown that Bph enzymes are very sensitive targets of inhibition by specific chlorinated TPs (e.g. chlorinated dihydroxybiphenyls - inhibiting BphB, chlorocatechols - inhibiting BphC chlorinated HOPDAs and benzoic acids - inhibiting BphD) (Dai et al., 2002; Martinez et al., 2007; Seah et al., 2000; Sondossi *et al.*, 1992; Zhang et al., 2009) that could trigger the activation of alternative biodegrading pathways. Since the identified chlorobenzoic acids suggested a full functionality of the upper *bph* pathway, we propose that the chlorobenzoic acids that started to accumulate in the growth medium were the trigger leading to the activation of this alternative pathway. However, we should not exclude the possibility that our four rhodococcal strains were able to use this alternative biotransformation pathway in parallel with the 2,3- and 3,4- dioxygenase pathway. As for the enzymes involved in this biotransformation, we hypothesized that enzymes from some other aromatic pathway might be responsible for these alternative PCB transformations. Rhodococci typically harbor an array of different pathways for the transformation of aromatics with many oxygenases and hydroxylases being functional homologues with redundant functions (Goncalves et al., 2006; Larkin et al., 2005; van Beilen et al., 2002) However, this finding would need additional support.

e. Accumulation of TPs containing pyridine moieties in assays with selected rhodococci

Different biphenyl derivatives containing nitrogen molecules (e.g. 2-phenylpyridine, phenyl 3-pyridyl-ketone, pyridyl-benzyl alcohol) and an array of TPs with two or more phenyl groups (e.g. 1,3-dichloro-8-hydroxynaphthalene, 2,4-dichloro-α-naphthol) were the most unusual TPs found in our study and were rather abundant in our experiments, but previously unreported in the literature. Even though the possibility of enzyme-assisted formation cannot be discarded, based on the structure of the identified TPs we postulated that: (i) TPs with two or more phenyl groups were generated through synthesis or polymerization of other TPs, while (ii) nitrogenous TPs were generated after an opening of the phenyl ring with N being incorporated into the molecule. Nitrogen-containing phenyls, identified as chloropicolinic acids, have been previously observed to be produced during transformation of mono-CB (Ahmad et al., 1991; Davison et al., 1999). As proposed, they can be derived from chlorobenzoic acids, catechols or HOPDA intermediates in the presence of ammonium. Additional studies are however necessary to determine if the formation of the mentioned TPs is spontaneous or a result of some biological catalysis.

Conclusion

Our results indicate an interplay between different catabolic pathway modules used by our rhodococcal isolates Z6, T6, Z57 and R2 in PCB transformation. This complex metabolic hub, which implied a metabolic flexibility within rhodococci, once more confirmed bacteria belonging to this genus as one of the most promising microorganisms for PCB transformation. The strategy of using diverse degrading pathways in a single microorganism could be essential for an effective bioremediation of PCBs to be realized. The analogous PCB transformation mechanisms found in rhodococci, originating from both terrestrial and marine environments, indicated a ubiquitous spread of such transformation systems in a wide

spectrum of environments. Likewise, our study clearly identified knowledge gaps still present when considering the biodegradation of these complex compounds and implied on the importance of considering other aromatic pathways that may function in their biotransformation. Further studies are needed to provide conclusive evidence of the existence of the proposed multiple systems for PCB-transformation in rhodococci, a feature that was always anticipated but never truly defined.

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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Unraveling metabolic flexibility of rhodococci in PCB transformation

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Abstract

Even though the genetic attributes suggest presence of multiple degradation pathways, most of rhodococci are known to transform PCBs only via regular biphenyl (bph) pathway. Using GC-MS analysis, we monitored products formed during transformation of 2,4,4'-trichlorobiphenyl (PCB-28), 2,2',5,5'-tetrachlorobiphenyl (PCB-52) and 2,4,3'-trichlorobiphenyl (PCB-25) by previously characterized PCB-degrading rhodococci Z6, T6, R2, and Z57, with the aim to explore their metabolic pleiotropy in PCB transformations. A striking number of different transformation products (TPs) carrying a phenyl ring as a substituent, both those generated as a part of the bph pathway and an array of unexpected TPs, implied a curious transformation ability. We hypothesized that studied rhodococcal isolates, besides the regular one, use at least two alternative pathways for PCB transformation, including the pathway leading to acetophenone formation (via 3,4 (4,5) dioxygenase attack on the molecule), and a third sideway pathway that includes stepwise oxidative decarboxylation of the aliphatic side chain of the 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate. Structure of the identified chlorinated benzoic acids and acetophenones allowed us to hypothesize that the first two pathways were the outcome of a ring-hydroxylating dioxygenase with the ability to attack both the 2,3(5,6) and the 3,4(4,5) positions of the biphenyl ring as well as dechlorination activity at both, -ortho and -para positions. We propose that several TPs produced by the bph pathway could have caused the triggering of the third sideway pathway. In conclusion, this study proposed ability of rhodococci to use different strategies in PCB transformation, which allows them to circumvent potential negative aspect of TPs on the overall transformation pathway.

Keywords: polychlorinated biphenyl, biotransformation, *Rhodococcus*, *bph* pathway, transformation products, multiple pathways

1. Introduction

PCBs, which comprises a family of 209 related compounds (congeners) and each with a distinct structure and environmental behaviour, are among the most notorious persistent organic pollutants (POPs)under the Stockholm convention (UNEP 2008). Unfortunately, today`s common perception of PCBs as "legacy" POPs is very misleading. Despite the fact that their manufacture was banned almost five decades ago, PCBs are still entering the environment, mainly *via* point source pollution, inadequately managed hazardous waste, leaching from electrical equipment or during waste treatment. Due to their environmental persistence and known ability for long distance migrations by natural atmospheric and oceanic processes (Ge et al., 2013), they are found all over the globe. By recycling throughout the environment, they often reach crops, animals and humans with concurrent bioaccumulation and biomagnification in the food web (Turrio-Baldassarri et al., 2007). In recent years, it has been conclusively shown that the so-called "non-Aroclor PCBs" also contaminate environment. These "non-legacy" PCBs represent unintentional by-products of manufacturing processes of certain pigments used in dyes, inks, and paints but can be unintentionally produced from any chemical process that involves C, Cl and elevated temperatures or catalysts (Grossman, 2013; Rudel and Perovich, 2009). Studies imply a complex picture of how both "non-legacy" PCBs alongside with the persisting "legacy" PCBs affect the environment and human health (Grossman, 2013).

Among an array of possible treatments for PCB disposal, the use of microbial resources for their degradation/transformation has been extensively studied and accepted as an eco-friendly and low-cost alternative. Various microorganisms belonging to diverse taxonomic groups (bacteria, cyanobacteria, and fungi) have been recognized as catabolically active in degrading/transforming PCBs (Xiang et al., 2020), including different bacterial genera

Pseudomonas, *Burkholderia*, *Comamonas*, *Cupriavidus*, *Sphingomonas*, *Acidovorax*, *Rhodococcus*, *Ralstonia*, *Bacillus*, cyanobacteria *Anabaena* or white rot fungi *Phanerochaete* (Abraham, 2002.; Bedard, 1986; Cvančarová et al., 2012; Garrido-Sanz et al., 2018; Komancova et al., 2003; Pieper, 2005; Sakai et al., 2002; Seto et al., 1995; Sharma et al., 2018; Zhang et al., 2015, Wang et al., 2018). Each of these microbial isolates shows quite unique spectra in regard to the type and extent of PCB congeners that they metabolize, with most, unfortunately, having rather narrow specificity, allowing the transformation of only a small number of PCBs (Arnett et al., 2000; Borja et al., 2005, McKay et al., 2003; Mondello et al., 1997). The genetic background for the observed PCB-degrading ability has been studied in many of these strains (Erickson et al. 1993; Hofer et al., 1994; Pieper, 2005), however, most of our knowledge on the PCBs biochemistry, metabolism and organization of catabolic genes still comes from model bacterial strains, *Paraburkholderia xenovorans* LB400, *Pseudomonas pseudoalcaligenes* KF707 and *Rhodococcus jostii* RHA1 whose genomes have been fully sequenced (Chain et al., 2006; Furukawa et al., 2008; McLeod et al., 2006)

The biphenyl (*bph*) catabolic pathway, presented in **Fig. 1,** has been identified as a main pathway through which PCBs are aerobically transformed by bacteria (Furukawa et al., 2004, Pieper, 2005). The pathway is initiated by the attack of biphenyl dioxygenase (BphA) on a 2,3(5,6) position of the, preferentially non-substituted or less substituted, biphenyl ring. The formed dihydrodiol is transformed in the second step by 2,3-dihydrodiol-2,3-dehydrogenase (BphB) to 2,3-dihydroxybiphenyl which is further cleaved by the enzyme 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) to yield a *meta*-cleavage product 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA). The last step, catalyzed by 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (BphD), generates two final metabolites of the upper *bph* pathway: benzoic acid and 2-hydroxypenta-2,4-dienoate. In addition to this pathway, it has

been found that certain bacteria can transform congeners that occupy the 2,3(5,6) positions of the ring, by oxygenation proceeding at 3,4 positions, with the proposed formation of acetophenones as the final metabolites (Bedard et al., 1990; Erickson et al., 1993; Gibson et al., 1993; Komancova et al., 2003). A complete and well-rounded understanding of this second PCB transformation pathway remains to be reached; however, PCB-pioneer researchers (Ahmad et al., 1991) have suggested several acidic metabolites found in culture extracts as intermediary metabolites leading to acetophenone formation.

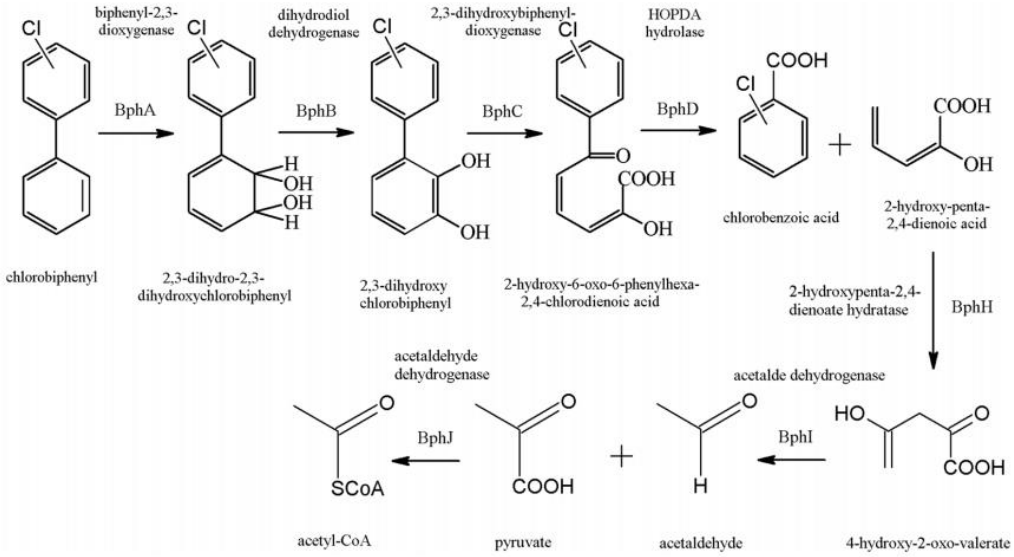

Fig. 1. The principal steps in *bph pathway* involved in PCBs microbial transformation. BphA (biphenyl-2,3-dioxygenase) catalyzes the transformation of biphenyl (1) to biphenyl-2,3-dihydrodiol (2). BphB (biphenyl-2,3-dihydrodiol-2,3-dehydrogenase) forms 2,3-dihydroxybiphenyl (3), BphC (2,3-dihydroxybiphenyl-1,2-dioxygenase) 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (4), and BphD (2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase) 2-hydroxypenta-2,4-dienoate (5a) and benzoic acid (5b).

It is clear that our knowledge on enzymes and pathways involved in the transformation of PCBs and its metabolites is still rather fragmentary given the pleiotropic strategies of microorganisms to transform PCBs. Already in 2008, Pieper and Seeger stated that the PCB metabolism should not be regarded as a simple linear pathway, but as a complex interplay between different catabolic pathway modules. As shown, depending on the congener itself,

and the specificity of the Bph enzymes harboured by a given organism, different *bph* pathway steps may constitute metabolic bottlenecks, resulting in the accumulation of the respective toxic metabolites which can be viewed as a kind of suicide inactivation of different Bph pathway enzymes (Dai et al., 2002; Garrido-Sanz et al., 2018). Bacterial strains using different pathways interplay, offering them a way to by-pass these issues, would have greater PCB-degrading ability. One such bacteria could be those belonging to the genus *Rhodococcus*, known for its high metabolic versatility in degrading different xenobiotics and by harbouring multiple and unique enzyme systems (Garrido-Sanz et al., 2020, Iwasaki et al., 2006; Masai et al. 1995; Seto et al. 1995; Taguchi et al., 2004; Van der Geize, 2004; Yang et al., 2004). During our previous studies on PCB-contaminated soils and marine sediments, we have successfully isolated several *Rhodococcus* strains capable of transforming a wide range of structurally different PCB congeners (Begonja Kolar et al., 2007, Petrić et al., 2007), a feature that could not be explained if only the known *bph* pathway was considered. Within the genome of several PCB-degrading rhodococci, *bph* genes encoding multiple pathway isoenzymes have already been identified (Pieper, 2005; Shimizu et al., 2001). Even so, information on the potential multiple pathway and complete array of intermediate metabolites produced during PCB transformation by rhodococci is still unavailable.

To obtain better insight into the PCB transformation mechanisms used by rhodococci, we designed an experiment in which we used gas chromatographic – mass spectrometric (GC-MS) analysis to monitor and identify transformation products (TPs) formed during the transformation of 3 selected (structurally) different PCB congeners: 2,4,4'-trichlorobiphenyl (PCB-28), 2,2',5,5'-tetrachlorobiphenyl (PCB-52) and 2,4,3'-trichlorobiphenyl (PCB-25). The potential wider spreading of the observed mechanisms within the *Rhodococcus* genus was studied by including four rhodococcal strains into the experiment, namely Z6, T6, R2, and Z57, isolated from different environments (Begonja Kolar et al., 2007, Petrić et al., 2007).

Under the presumption that the full catabolic potential of rhodococci in PCB biodegradation is yet to be discovered, we aimed to determine the level of specificity of the enzymes involved in PCB biotransformation, having in mind the potential of our study to unravel an existence of multiple PCB-degrading pathways in rhodococci.

2. Materials and methods

2.1. Bacterial strains, culture media and chemicals used

The PCB-degrading bacterial strains used in the study included *Rhodococcus* isolates Z6, T6, R2, and Z57, with details on each isolate provided in **Table 1** (Begonja Kolar et al., 2007, Petrić et al., 2007). As a basal medium, we used phosphate-buffered minimal salts medium (PAS), both for growth of the strains and for biodegradation experiments (Bedard et al., 1986). Its modified version was used for the strains originating from marine sediments (MPAS) by mixing filter sterilized seawater and PAS medium at 1:1 (Begonja Kolar et al., 2007). The PCB congeners, 2,4,4'-trichlorobiphenyl (2,4,4'-CB), 2,2',5,5'-tetrachlorobiphenyl (2,2',5,5'-CB) and 2,4,3'-trichlorobiphenyl (2,4,3'-CB), used in the study were purchased from Sigma-Aldrich as BCR® certified Reference Materials. Stock solutions of individual congeners were prepared in acetone (1mg/ml). Biphenyl ($C_6H_5C_6H_5$; 99.5 % purity) and other high purity chemicals used for the extraction of metabolites and GC-MS analysis were purchased from Sigma-Aldrich and Merck (Darmstadt, Germany).

152 **Table 1.** Information on the PCB degrading *Rhodococcus* strains used in the study.

Label	16S rRNA gene identity	Enriched from the mixed culture	Mixed culture origin [PCB mass fraction in sample (mg kg^{-1})][a]	Sampling location
Z6	*Rhodococcus* sp.[b]	TSZ7	Transformer station soil [6044]	Zadar, Croatia
T6	*Rhodococcus* sp.[b]	AIR1	Airport soil [2.210)	Trogir, Croatia
R2	*Rhodococcus erythropolis*[c]	RMC2	Harbour marine sediment [0.495]	Rijeka, Croatia
Z57	*Rhodococcus ruber*[c]	ZMC57	Harbour marine sediment [0.540]	Zadar, Croatia

153 [a] PCB mass fraction in sample (mg kg^{-1}) determined against Aroclor 1248+1254 standards
154 [b] Petrić et al., 2007
155 [c] Begonja Kolar et al., 2007

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157 ***2.2. PCB biodegradation assays***

158 Biodegradation assays were performed in 50-mL Erlenmeyer flasks containing (i) 20 ml
159 of sterile PAS or MPAS medium, (ii) the selected individual PCB congener, provided at a
160 final concentration of 5 mg l^{-1}, (iii) the biphenyl, used as an inducer, provided at a final
161 concentration of 250 mg l^{-1}, and (iv) 2 ml of individual bacterial inoculum. Starter bacterial
162 inoculums (3-day old cultures) were prepared for the experiment by growing selected
163 *Rhodococcus* strains in PAS or MPAS medium supplemented only with biphenyl at a
164 concentration of 1 g l^{-1}. Flasks without bacterial inoculums were likewise included in the
165 assay, representing controls. These controls allowed us to follow the potential abiotic
166 transformation of the selected PCB congeners. The prepared flasks were incubated on a
167 rotary shaker at 200 rpm and 28 ^{o}C, under aerobic conditions (flasks were plugged with
168 cotton pads). Biodegradation assay was conducted over seven days. All assays were
169 performed in duplicates.

The PCB transformation potential of strains Z6, T6, R2 and Z57 was investigated using two model congeners: (i) 2,4,4'-CB, with all 3,4 ring positions blocked, chosen as a model to follow the attack of BphA on the free 2,3 position, and (ii) 2,2',5,5'-CB, with all 2,3 ring positions blocked, chosen as a model to follow the attack of BphA on a 3,4 position. In addition, the potential of strains R2 and Z57 to transform congener 2,4,3'-CB was also investigated, both having free positions for a 2,3 and 3,4 attack.

2.3. Gas chromatographic- mass spectrometric analysis

To assure an accurate chemical analysis of compounds (PCBs, TPs), we decided to process the whole volume of the sample and therefore samples were taken for the GC-MS analysis only at the end of the experiment (at day 7). This was required due to the hydrophobic properties of PCBs, e.g. potential sorption. Hence, whole flask volumes were submitted to the extraction procedure described previously in detail (Petrić et al., 2007). Briefly, two organic layers obtained from neutral extraction (with dichloromethane) and acid extraction (with dichloromethane and hydrochloric acid) were joined. After drying over anhydrous sodium sulfate and concentration by a gentle stream of nitrogen, extracts were subjected to GC-MS using a GC/MS HP Agilent instrument (Palo Alto,CA, USA). The instrument was equipped with a DB-5MSITD column (30 m x 0.25 mm, film thickness 0.25 μm) (J&W Scientific, Folsom, USA) with helium used as a carrier gas. The column was heated from 35 ^{o}C (1min hold) to 300 ^{o}C at 5 ^{o}C min^{-1} rate (15 min hold). Mass spectra of the transformation products were recorded in the full scan acquisition mode (mass range 33-650 *m*/*z*) and the scan rate of 1 scan s^{-1}. The injection volume of the sample was 2 μl. Given that the vast majority of the TPs did not exist within database libraries (NIST, Wiley), their identification was performed by a comprehensive examination of the fragmentation patterns of the mass spectra ions.

3. Results

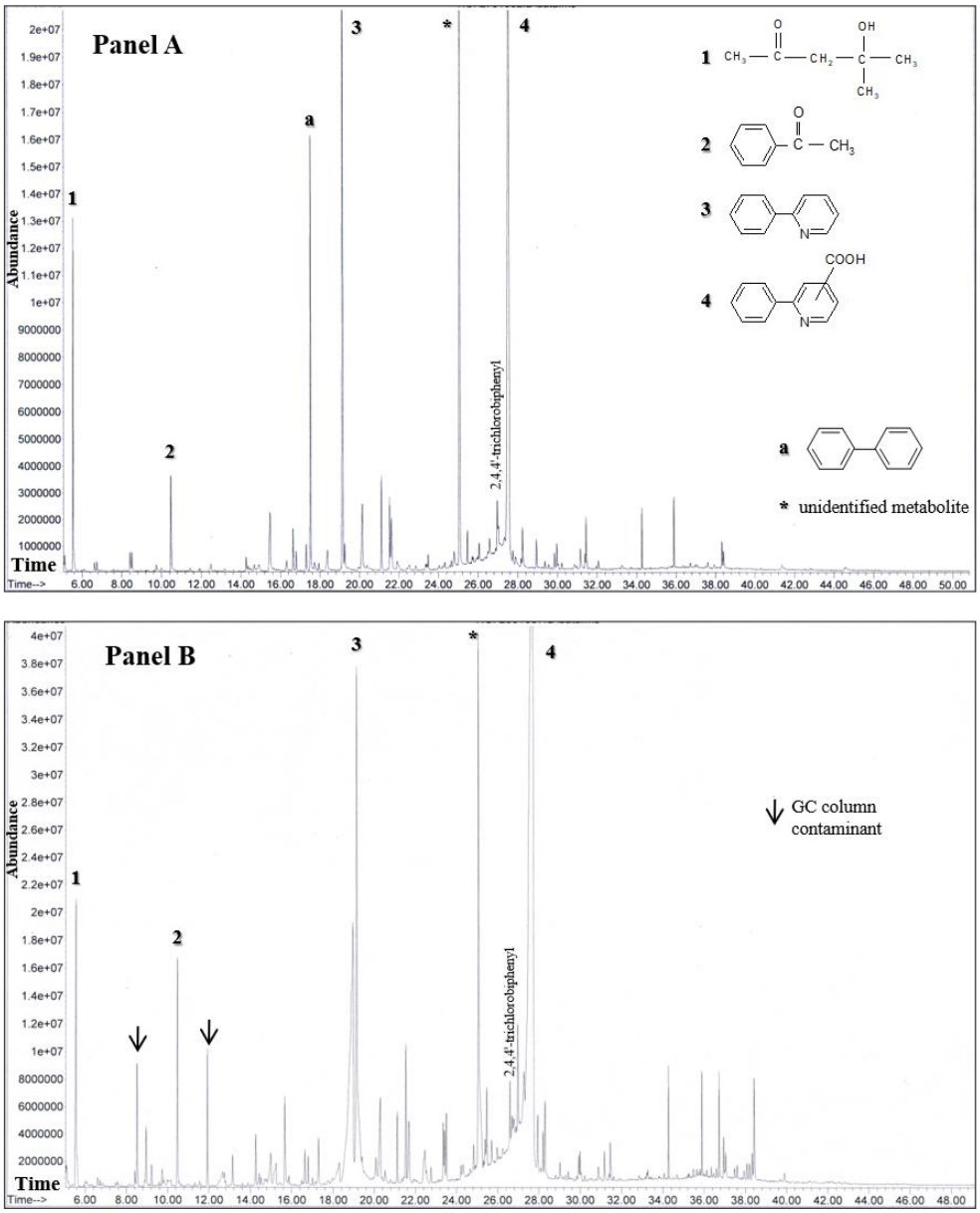
A visual examination of the flasks at the end of the 7-day biodegradation experiment suggested that all 4 tested rhodococci successfully grew in the presence of a biphenyl (observed turbidity), while an intensive yellow color (indication of the *meta*-cleavage product HOPDA) suggested biotransformation of biphenyl and/or model congeners. Biotic transformation of the compounds was further confirmed by (i) visual examination of the control flasks in which no changes in the turbidity and/or color were observed and (ii) GC-MS analysis of the control flasks in which both the originally added biphenyl and individual PCB congener were detected in the extracts. In addition, we detected one biphenyl transformation product (with an additional methyl group on one phenyl ring) in the controls, which was presumed to represent an impurity of the biphenyl used in the experiment.

3.1. Transformation of 2,4,4'-CB by the selected rhodococci

Total ion current GC-MS chromatograms of the culture extracts obtained for all rhodococcal strains at the end of the experiment with 2,4,4'-CB are presented in **Figs. 2A** and **2B**, while the list and presumed formulas of the identified TPs are given in **Tables 2A** and **2B.** In addition, the mass spectra of some of the key TPs detected in this assay are provided in the Supplementary material (**Fig S1**). Residual amounts of the originally added biphenyl and 2,4,4'-CB congener (peak with symbol "a" and peak "2,4,4'-trichlorobiphenyl", **Fig. 2A**) indicated that the transformation process was still not finished at day 7. Still, the formation of a number of TPs was commonly observed in extracts of all rhodococcal isolates. They included both non-chlorinated biphenyl TPs (**Table 2A**), but likewise an array (up to 19 depending on the strain) of chlorinated TPs carrying a phenyl ring as a molecule substituent (**Table 2B**). From an array of different chlorinated TPs, tagged as those deriving from 2,4,4'-CB, only 4 TPs were shared in all 4 tested rhodococci and included dichlorinated benzoic

218 acid (CB2) and acetophenone (CB3), 2,4-dichlorophenylacetic acid (CB7) and 3-(2,4-
219 dichlorophenyl)-2-propenoic acid (dichlorinated cinnamic acid) (CB10). Other TPs included
220 an array of different phenyl compounds with an aliphatic side chain containing from 1 to 5 C
221 atoms: (i) carboxylic acids R–COOH (such as phenylpropenoic (cinnamic) and propanoic
222 acids or phenyl acetic acids) or (ii) alcohols R-OH (such as phenol, phenyl methanols or
223 phenyl ethanoles).

224 Interestingly, apart from the expected TPs carrying a phenyl ring as a substituent, an
225 array of additional TPs was identified in the analyzed extracts. One was a linear metabolite,
226 identified as 4-hydroxy-4-methyl-2-pentanone (peak no. 1, **Figs. 2A** and **2B**) detected in all
227 of the culture extracts. In addition, unexpected TPs included those containing pyridine
228 moieties as well as TPs built up of up to three phenyl rings (**Fig. 3**), identified in all culture
229 extracts (such as 2-phenyl pyridine, 2-phenyl-3-hydroxypyridine, 2-phenylpyridine
230 carboxylic acid, 2-(2,4-dichlorophenyl) pyridine, 2-(2,4-dichlorophenyl)-5-chloropyridine,
231 2,4-dichlorophenyl-4-chlorophenyl-ketone, di-(2,4-dichlorophenyl)-ketone). Many of these
232 unexpected peaks also represented the most prominent TPs standing out from the others in
233 GC-MS chromatograms by their peak intensities (peak heights) (peak nos. 1, 3, 4, 6, 7 and 9,
234 **Figs. 2A and 2B**). High intensity TP, eluting from the column around minute 25, was not
235 positively identified (peak with an asterisk symbol, **Figs. 2A** and **2B**). In the assays with
236 strains Z6, T6 and R2, one of these prominent TPs corresponded to non-chlorinated
237 acetophenone (peak no. 2, **Figs. 2A** and **2B**). Compared to strains Z6 and T6, chromatograms
238 of strains R2 and Z57 included greater numbers of high intensity peaks. Unfortunately, with
239 no standards available, it was not possible to determine the actual concentration of each of the
240 detected TPs.



241

242 **Fig. 2A.** Full-scan GC-MS chromatograms of the culture extracts after 7-day of incubation of
243 Rhodococcus sp. Z6 (panel A) and Rhodococcus sp. T6 (panel B) with 2,4,4'-trichlorobiphenyl in the
244 presence of biphenyl.

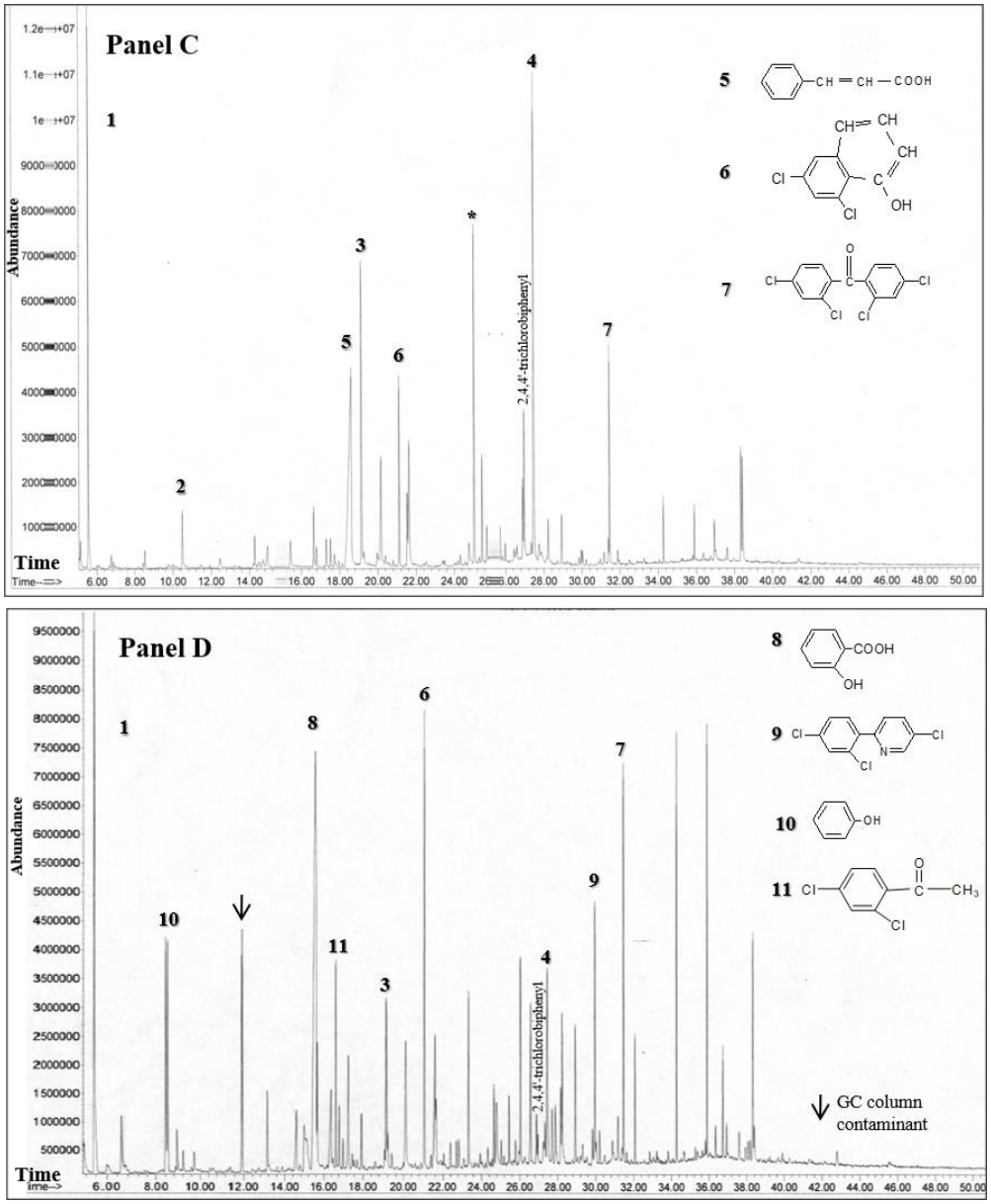
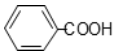
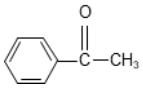
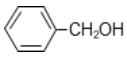
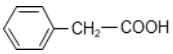
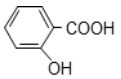
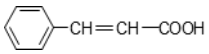
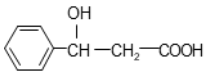
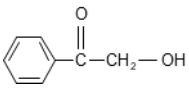


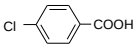
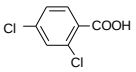
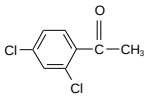
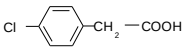
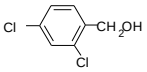
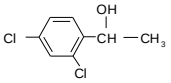
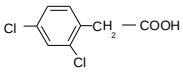
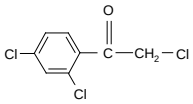
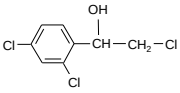
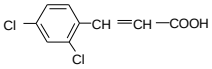
Fig. 2B. Full-scan GC-MS chromatograms of the culture extracts after 7-day incubation of *Rhodococcus erythropolis* R2 (Panel C) and *Rhodococcus ruber Z57* (Panel D) with 2,4,4'-trichlorobiphenyl in the presence of biphenyl

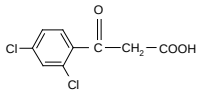
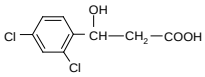
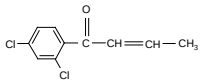
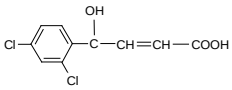
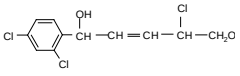
Table 2A. Identification and mass spectra of the TPs formed from biphenyl during 2,4,4'-trichlorobiphenyl biodegradation in cultures of *Rhodococcus* Z6, T6, R2 or Z57.

Transformation product (TP) designation	TP	TP formula	TP *m/z*[a]	Microbial culture
B1	Benzoic acid		122,105,77,51	Z6, T6, R2
B2	Acetophenone		120,105,77,51	Z6, T6, R2
B3	Phenol		94,66,50	all
B4	Benzyl alcohol		108,91,79,51	all
B5	2-phenylacetic acid		39,65,91,136	Z6, Z57
B6	Salicylic acid		64,92,120,138	Z6, R2, Z57
B7	Cinnamic acid		147,130,103,77	Z6, T6, R2
B8	3-hydroxy-3-phenylpropanoic acid		166,107,79,51	Z6, T6, R2
B9	2-hydroxy-1-phenyl-ethanone		51,77,105,136	Z6

[a] Main mass spectra *m/z* ions

Table 2B. Identification and mass spectra of the chlorinated TPs formed from 2,4,4'-trichlorobiphenyl during biodegradation in cultures of Rhodococcus Z6, T6, R2 and Z57.

Transformation product (TP) designation	TP	TP formula	TP *m/z*[a]	Microbial culture
CB1	**4-chlorobenzoic acid**		50,75,111,139,156	Z6, Z57
CB2	**2,4-dichlorobenzoic acid**		74,109,145,173,190	all
CB3	**2,4-dichloroacetophenone**		75,109,145,173	all
CB4 2C[b]	4-chlorophenylacetic acid		44,92,170,125,44	Z57
CB5 2C[b]	2,4-dichlorophenyl methanol		51,77,111,141,176	Z6, R2
CB6 2C[b]	α-methyl-2,4-dichlorophenyl methanol		43, 75,111,147,175	Z6, Z57
CB7 2C[b]	2,4-dichlorophenylacetic acid		63,89,125,159,204	all
CB8 2C[b]	2-chloro-1-(2,4-dichlorophenyl)ethanone		75,109,145,173	Z6, T6
CB9 2C[b]	2,4-dichloro-α-(chloromethyl)benzenemethanol		50,75,111,147,175	Z6, R2
CB10 3C[b]	3-(2,4-dichlorophenyl)-2-propenoic acid		74,99,125,136,181,216	all

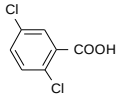
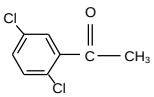
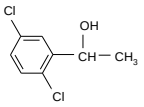
CB11 3C[b]	3-(2,4-dichlorophenyl)-3-oxo-propanoic acid		232,216,173,145	R2, Z57
CB12 3C[b]	1-hydroxy-1-(2,4-dichlorophenyl)-2-propanoic acid		232,214,198,175	R2
CB13 4C[b]	1-(2,4-dichlorophenyl)2-butene-1-on		111, 173,145	Z6, T6
CB14 4C[b]	4-(2,4-dichlorophenyl)-4-hydroxy-2-butenoic acid		230,195,175,56	Z57
CB15 5C[b]	5-(2,4-dichlorophenyl)-5-hydroxy-2-chloropentenol		282,252,188,175	R2, Z57

[a] Main mass spectra *m/z* ions
[b] Number of C in the aliphatic side chain

3.2. Transformation of 2,2',5,5'-CB by the selected rhodococci

In comparison with the previously studied congener, a much smaller number of peaks was detected in GC-MS chromatograms of the 2,2',5,5'-CB biodegradation media at the end of the experiment (**Fig S2**), with strain Z57 characterized by the smallest number of detected TPs. Some of the TPs were identical to those found in the 2,4,4'-CB assay and included non-chlorinated compounds formed from biphenyl: (i) B1 (benzoic acid), (ii) B2 (acetophenone), (iii) B4 (benzyl alcohol) and (iv) B7 (cinnamic acid), identified in Z6, T6 and R2 assays, and in addition (v) B6 (salicylic acid), identified in the assay of strain R2 (**Table 2A**). From chlorinated TPs, carrying a phenyl ring as a molecule substituent, we identified only 3 compounds (**Table 3**): (i) dichlorinated benzoic acid (CB16), (ii) dichloroacetophenone (CB17) and (iii) α-methyl-2,5-dichlorobenzyl alcohol (CB18). Once more, in all culture extracts, an unexpected linear TP (4-hydroxy-4-methyl-2-pentanone) and TPs containing pyridine moieties (such as 2-phenylpyridine and/or 2-phenylpyridine-carboxylic acid) were identified (**Fig S2**). High residual amounts (high peaks) of the originally added biphenyl and 2,2',5,5'-CB were detected at the end of the experiment (**Fig S2**). Higher prevalence of the following, above mentioned TPs, was recorded in the culture extracts: 4-hydroxy-4-methyl-2-pentanone, non-chlorinated acetophenone (B2), cinnamic acid (B7) and 2,5-dichlorobenzoic acid (CB16) (**Fig S2**).

277 **Table 3.** Identification and mass spectra of chlorinated TPs formed from 2,2',5,5'-tetrachlorobiphenyl
278 during biodegradation in cultures of Rhodococcus Z6, T6, R2 and Z57. ***** Metabolites marked as B1,
279 B2, B4 and B7 presented in the Table 2 were also identified in culture extracts of isolates Z6, T6 and
280 R2. In addition metabolite B6 was identified in the R2 isolate culture extracts.

Transformation product (TP) designation	TP	TP formula	TP *m/z*[a]	Microbial culture
CB16	**2,5-dichlorobenzoic acid**		74,109,145,173,190	Z6, T6, R2
CB17	**2,5-dichloroacetophenone**		71,145,175,188	all
CB18 2C[b]	α-methyl-2,5-dichlorobenzyl alcohol		111,147,175,190	all

281 [a] Main mass spectra *m/z* ions
282 [b] Number of C in the aliphatic side chain
283

284 ### *3.3. Transformation of 2,4,3'-CB by the selected rhodococci*

285 The transformation activity towards congener 2,4,3'-CB was tested only on two of the
286 selected isolates, Z57 and R2 (**Fig S3**) with an identified small number of non-chlorinated
287 and chlorinated TPs, mostly analogous to those detected in the biodegradation assays with
288 either 2,4,4'-CB or 2,2',5,5'-CB congener. We found relatively high residual amounts of the
289 originally added congener 2,4,3'-CB in both culture extracts. The TPs identified in the culture
290 extract included non-chlorinated TPs (i) B2 (acetophenone) and (ii) B8 (3-hydroxy-3-
291 phenylpropanoic acid), (iii) dichlorinated acetophenone (CB3), (iv) α-methyl-2,4-
292 dichlorophenyl methanol (CB6) and (v) 2-phenylpyridine, with the formula already presented

293 in **Tables 2A** and **2B** and in **Fig. 3**. The three last TPs represented peaks with the highest
294 intensity in the GC-MS chromatograms (**Fig S3**). When compared to R2, a new TP - 2,4-
295 dichlorobenzaldehyde (*m/z* 75,111,145,173; in 11.45 min) was identified in the culture
296 extracts of the isolate Z57.

297 **4. Discussion**

298 The PCB metabolism in rhodococci is presumed to be a complex interplay between
299 different catabolic pathway modules, however, clear information on these pathways still
300 remains to be attained. In our study, we focused on identifying all of the TPs produced during
301 the biotransformation of PCBs by 4 studied rhodococcal strains, and based on the key TPs
302 detected (mainly in the assay with 2,4,4'-CB), we proposed tentative pathways used by
303 rhodococci for PCB transformation (**Fig. 4**). As explained in more details in the following
304 paragraph of the discussion, these pathways did not rely merely on the regular *bph* pathway
305 leading to chlorobenzoic acids formation (**Fig. 4,** Scheme upper part, step 1a and 1b,
306 Paragraph 4.1), but also included additional pathways: benzoic acids formation (i) via
307 stepwise oxidative decarboxylation of the aliphatic side chain (**Fig. 4,** Scheme upper part,
308 step 2, explained in Paragraph 4.4.) and (ii) via intradiol opening of the biphenyl ring (**Fig. 4,**
309 Scheme upper part, step 3, explained in Paragraph 4.4.**)** and also pathway leading to
310 acetophenone formation (**Fig. 4,** Scheme lower part, explained in Paragraph 4.3.). The
311 existence of multiple pathways in single strains has up until now been proposed only in
312 *Rhodococcus jostii* RHA1 (Iwasaki et al., 2006) and *Rhodococcus* sp. WAY2 (Garrido-Sanz
313 et al., 2018).

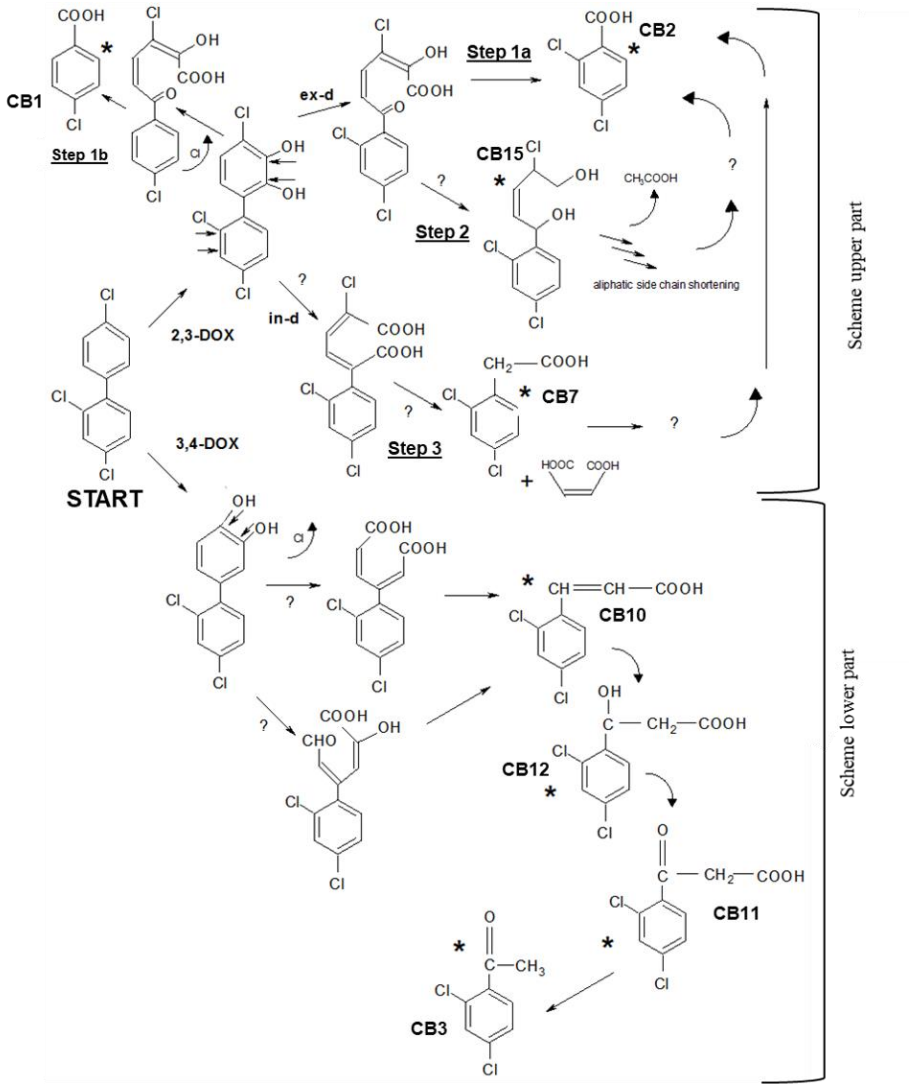


Fig. 4. Tentative pathways proposed to be active in rhodococci during biotransformation of congener 2,4,4`-CB (START):

(1) 2,3-dioxygenation (2,3-DOX) of the biphenyl ring (upper part of the scheme)

- *regular bph pathway* leading to the production of 2,4-dichlorobenzoic acid (CB2) **(Step 1a)** and 4-chlorobenzoic acid (CB1) (**Step 1b).**
- *alternative patway* working *via* shortening of the HOPDA aliphatic side chain (**Step 2**) with 5–(2,4-dichlorophenyl)-5-hydroxy-2-chloropentenol (CB15) as one of the TP, leading to the production of 2,4-dichlorobenzoic acid (CB2).
- *alternative pathway* working via intradiol opening of the biphenyl ring (**Step 3**) with 2,4-dichlororphenylacetic acid (CB7) as one of the TP, leading to the production of 2,4-dichlorobenzoic acid (CB2).

(2) 3,4-dioxygenation (3,4-DOX) of the biphenyl ring (lower part of the scheme)

- *alternative pathway* leading to the production of 2,4-dichlororacetophenone (CB3) with dichlorinated cinnamic acid (CB10), 3-(2,4-dichlorophenyl)-3-oxo-propanoic acid (CB11) and 1-hydroxy-1-(2,4-dichlorophenyl)-2-propanoic acid (CB12) as TPs.

* TPs identifed in the assay with 2,4,4`-CB with details provided in Table 2B
in-d = intradiol opening
ex-d = extradiol opening

334 ***a. Existence of regular pathway leading to chlorobenzoic acids formation***

335 Identification of chlorinated benzoic acids in the culture extracts confirmed that our
336 rhodococci followed a *bph* pathway initiated by the primary dioxygenase attack on the
337 2,3(5,6) positions of the biphenyl ring. Although the less chlorinated ring was shown to be
338 more susceptible to bacterial transformation, strains Z6 and Z57 showed additional ability to
339 attack both rings of the 2,4,4'-CB congener, as evident from the detection of both di- and
340 mono-chlorinated benzoic acids (**Fig. 4,** step 1a and 1b). In addition, the unexpected
341 detection of chlorinated benzoic acids also in the assay with congener 2,2',5,5'-CB, with no
342 required free 2,3(5,6) positions for the initial enzyme attack, indicated very flexible and
343 unusual dioxygenase harboured by our rhodococcal strains. Based on the available literature
344 we propose two hypotheses for the observed benzoic acids formation in assay with 2,2',5,5'-
345 CB: (i) dechlorination of one of the phenyl rings at -*ortho* position could have preceded 2,3-
346 dihydroxylation, after which this congener followed the regular *bph* pathway or (ii) benzoic
347 acids were formed after an initial attack at free 3,4(5,6) positions following a non-regular
348 pathway - *via* the production of chlorinated cinnamic acid as an intermediate TP. This
349 seldomly reported pathway (Ahmad et al., 1991; Hilton and Cain 1990) proposes a chain of
350 reaction that includes the transformation of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (or
351 its saturated analogues) to chlorinated cinnamic acid and then to chlorinated benzoic acid.
352 Chlorinated cinnamic acids, even though detected in the assay with 2,4,4'-CB, were not
353 identified in the assay with 2,2',5,5'-CB congener, therefore preventing us from reaching a
354 firm final conclusion on one of these hypotheses. Of the four tested rhodococci, only strain
355 Z57 was not shown to possess this transformation ability.

356 Chlorobenzoic acids, formed at the end of the known upper *bph* pathway, are generally
357 further transformed very slowly by PCB-degrading bacteria, making this step a rate limiting

stage in the overall PCB-biodegradation (Zhang et al., 2009). Based on some of the identified TPs (e.g. phenols, benzyl alcohols and 2,4-dichlorophenylmethanol), we propose that benzoic acids are not necessary dead-end intermediates in PCB transformation but are further transformed *via* initial 1,2-dioxygenation (Davison et al., 1999). The latter two of these TPs have been previously associated with the transformation of chlorinated benzoic acids by ligninolytic fungi (Muzikár et al., 2011).

b. The existence of an alternative pathway leading to acetophenones formation

Specificity of dioxygenase harboured by rhodococcal strains

The identification of chlorinated acetophenones in the culture extracts suggested that an alternative pathway was also active in our rhodococcal isolates. As proposed by other authors, if the chlorine atoms are blocking the 2,3(5,6) positions, oxidation of the biphenyl ring occurs *via* a 3,4(4,5) attack, with acetophenones arising as the final TPs (Bedard et al., 1990; Erickson et al., 1993; Gibson et al., 1993; Komancova et al., 2003). Following this scheme we expected to detect acetophenones only in assays with congeners 2,2',5,5'-CB and 2,4,3'-CB; however, we likewise found them to be formed from 2,4,4'-CB. This indicated the specific nature of dioxygenase harboured by all four rhodococci able (i) to attack both 2,3(5,6) and 3,4(4,5) positions of the biphenyl rings but likewise (ii) to dechlorinate PCB molecules. The studied rhodococcal strains might either harbor very specific Biphenyl dioxygenase or the observed activity could be due to a combined action of different ring-hydroxylating dioxygenase as part of the *etb* (ethylbenzene) *ben* (benzoate), *cat* (catechol), *pca* (protocatechuate) or *nar* (naphthalene) pathways, known to have overlapping functions (Garrido-Sanz et al., 2018; Iwasaki et al., 2006; Kimura et al., 2006; Kitagawa et al., 2001a and 2001b; Sakai et al., 2003).

The mechanisms previously proposed (Bedard et al., 1990; Haddock et al., 1995,) indicate that ring-hydroxylating dioxygenases might concurrently also conduct dechlorination. Based on the chlorine substitution patterns of the congeners used in the assays, we propose that our rhodococci holds the dechlorination ability for the -*para* and -*ortho* position of the biphenyl ring. PCB-degraders usually do not have the ability to also dehalogenate PCBs, but several successful strains, including some rhodococci, can be found throughout the literature (Ahmad et al., 1991 ; Haddock et al., 1995 ; Komancovà et al., 2003 ; Rybkina et al., 2003; Pieper, 2005 ; Yang et al., 2004). The proposed dechlorination ability further supports the first hypothesis postulated earlier about the formation of chlorinated benzoic acids in the assay with congener 2,2',5,5'-CB.

Interestingly, the identification of non-chlorinated acetophenone, presumed to be formed from biphenyl, further implied that the proposed alternative pathway is not activated in the case of blocked 2,3(5,6) positions, but rather acts as a parallel pathway. The specificity of rhodococcal dioxygenases were further revealed in the assay with a third model congener 2,4,3'-CB. The preferential detection of dichlorinated acetophenone over benzoic acid indicated that our rhodococci might follow an alternative pathway for PCB transformation rather than the regular one.

c. The proposed pathway leading to acetophenone formation

Identification of certain "acidic" TPs in the culture extracts and the available, but scarce literature reports (Hilton and Cain, 1990), allowed us to propose part of the alternative pathway leading to the formation of the identified acetophenones (**Fig 4,** lower part of the Scheme). We propose that chlorinated "acidic" TPs [dichlorinated cinnamic acid – CB10, 3-(2,4-dichlorophenyl)-3-oxo-propanoic acid – CB11 and 1-hydroxy-1-(2,4-dichlorophenyl)-2-propanoic acid – CB12] take part in the alternative pathway, all preceding the formation of

acetophenone. Unfortunately, due to the small number of TPs identified in the assays with congeners 2,2',5,5'-CB and 2,4,3'-CB, these conclusions are only based on the data obtained with congener 2,4,4'-CB. However, it was evident that the formation of these unexpected "acidic" TPs, obviously not part of the regular *bph* pathway, was not limited to PCB transformation but was also used simultaneously by our four rhodococci for biphenyl transformation (detection of non-chlorinated "acidic" TPs: cinnamic acid and 3-hydroxy-3-phenylpropanoic acid). Finally, the high intensities of peaks corresponding to TPs such as acetophenones (non- and di-chlorinated) and cinnamic acid implicated the importance of this alternative pathway in PCB transformation by our rhodococcal strains.

d. The existence of an additional transformation pathway for chlorobenzoic acid formation

Additionally, the spectrum of the identified phenylic compounds carrying an aliphatic side chain containing two to five carbon atoms (different carboxylic acids R–COOH and alcohols R-OH) that could not be attributed to neither regular *bph* pathway nor the proposed alternative pathway, indicated the existence of even more pathway(s) for PCB transformation by the studied rhodococci. These types of compounds can scarcely be discovered in the literature and have up until now been detected only in several PCB-biodegradation assays conducted with *Pseudomonas, Achromobacter* and *Bacillus* (Ahmad et al. 1991; Masse et al., 1989). Based on this study, we propose that these phenylic compounds carrying an aliphatic side chain are not dead-end intermediates generated by the spontaneous cleavage of some unstable intermediate, but are generated by the *meta*-cleavage of the HOPDA, formed by the regular *bph* pathway. We propose that this third pathway involved stepwise oxidative decarboxylation of the aliphatic side chain of the HOPDA (**Fig. 4**, step 2) leading finally to alternative way of chlorobenzoic acid formation**.** As similarly concluded by Ahmed et al.

(1991), some bacterial strain do not necessarily convert HOPDA directly to benzoic acid but *via* its saturated analogues. Curiously, the identification of one specific TP, namely 2,4-dichlorophenylacetic acid (CB7, **Fig. 4**), allowed us to speculate about the possibility of a fourth additional pathway leading to the formation of chlorobenzoic acids, which occurred *via* an intradiol opening of the biphenyl ring (**Fig. 4**, step 3).

This experiment, unfortunately, did not allow us to define how this pathway is regulated i.e. if an alternative pathway functions in parallel from the beginning of the transformation process or is activated at a later step during the transformation. Studies have clearly shown that Bph enzymes are very sensitive targets of inhibition by specific chlorinated TPs (e.g. chlorinated dihydroxybiphenyls - inhibiting BphB, chlorocatechols - inhibiting BphC chlorinated HOPDAs and benzoic acids - inhibiting BphD) (Dai et al., 2002; Martinez et al., 2007; Seah et al., 2000; Sondossi *et al.*, 1992; Zhang et al., 2009) that could trigger the activation of alternative biodegrading pathways. Since the identified chlorobenzoic acids suggested a full functionality of the upper *bph* pathway, we propose that the chlorobenzoic acids that started to accumulate in the growth medium were the trigger leading to the activation of this alternative pathway. However, we should not exclude the possibility that our four rhodococcal strains were able to use this alternative biotransformation pathway in parallel with the 2,3- and 3,4- dioxygenase pathway. As for the enzymes involved in this biotransformation, we hypothesized that enzymes from some other aromatic pathway might be responsible for these alternative PCB transformations. Rhodococci typically harbor an array of different pathways for the transformation of aromatics with many oxygenases and hydroxylases being functional homologues with redundant functions (Goncalves et al., 2006; Larkin et al., 2005; van Beilen et al., 2002) However, this finding would need additional support.

e. Accumulation of TPs containing pyridine moieties in assays with selected rhodococci

Different biphenyl derivatives containing nitrogen molecules (e.g. 2-phenylpyridine, phenyl 3-pyridyl-ketone, pyridyl-benzyl alcohol) and an array of TPs with two or more phenyl groups (e.g. 1,3-dichloro-8-hydroxynaphthalene, 2,4-dichloro-α-naphthol) were the most unusual TPs found in our study and were rather abundant in our experiments, but previously unreported in the literature. Even though the possibility of enzyme-assisted formation cannot be discarded, based on the structure of the identified TPs we postulated that: (i) TPs with two or more phenyl groups were generated through synthesis or polymerization of other TPs, while (ii) nitrogenous TPs were generated after an opening of the phenyl ring with N being incorporated into the molecule. Nitrogen-containing phenyls, identified as chloropicolinic acids, have been previously observed to be produced during transformation of mono-CB (Ahmad et al., 1991; Davison et al., 1999). As proposed, they can be derived from chlorobenzoic acids, catechols or HOPDA intermediates in the presence of ammonium. Additional studies are however necessary to determine if the formation of the mentioned TPs is spontaneous or a result of some biological catalysis.

Conclusion

Our results indicate an interplay between different catabolic pathway modules used by our rhodococcal isolates Z6, T6, Z57 and R2 in PCB transformation. This complex metabolic hub, which implied a metabolic flexibility within rhodococci, once more confirmed bacteria belonging to this genus as one of the most promising microorganisms for PCB transformation. The strategy of using diverse degrading pathways in a single microorganism could be essential for an effective bioremediation of PCBs to be realized. The analogous PCB transformation mechanisms found in rhodococci, originating from both terrestrial and marine environments, indicated a ubiquitous spread of such transformation systems in a wide

spectrum of environments. Likewise, our study clearly identified knowledge gaps still present when considering the biodegradation of these complex compounds and implied on the importance of considering other aromatic pathways that may function in their biotransformation. Further studies are needed to provide conclusive evidence of the existence of the proposed multiple systems for PCB-transformation in rhodococci, a feature that was always anticipated but never truly defined.

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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Declaration of interests

☒ The authors Petrić, Ines, Drevenkar, Vlasta, Fingler, Sanja, Begonja Kolar, Ana, Hršak, Dubravka, Martin-Laurent, Fabrice and Udiković-Kolić, Nikolina declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

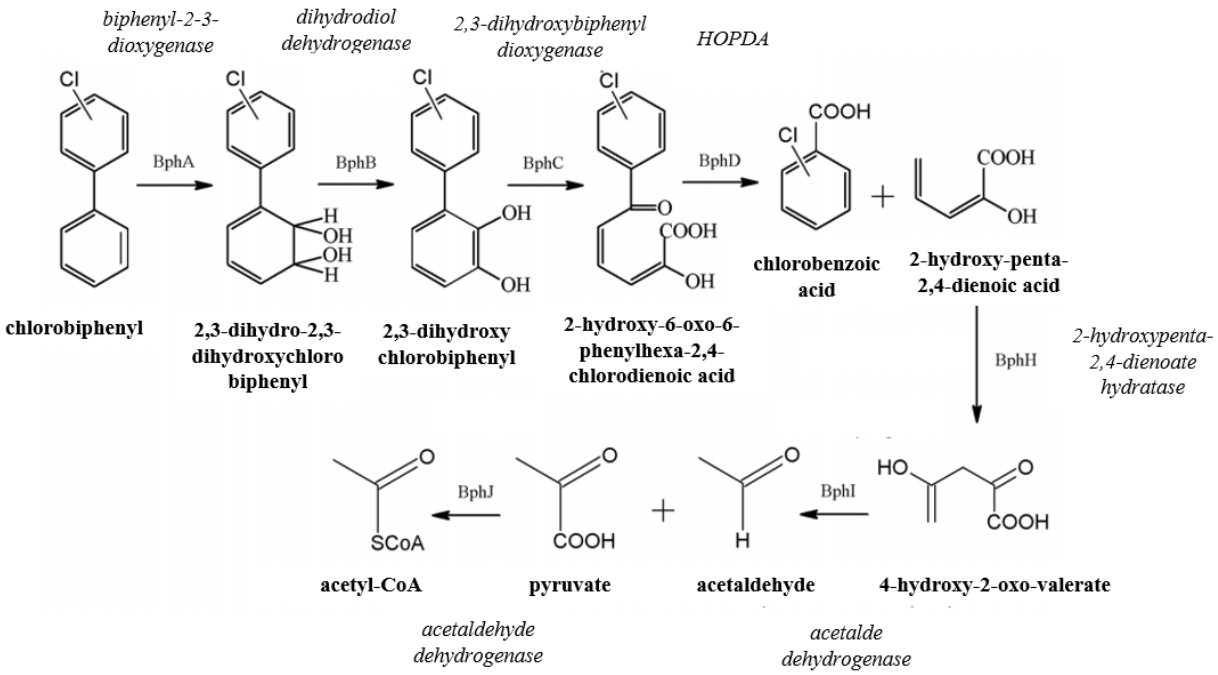
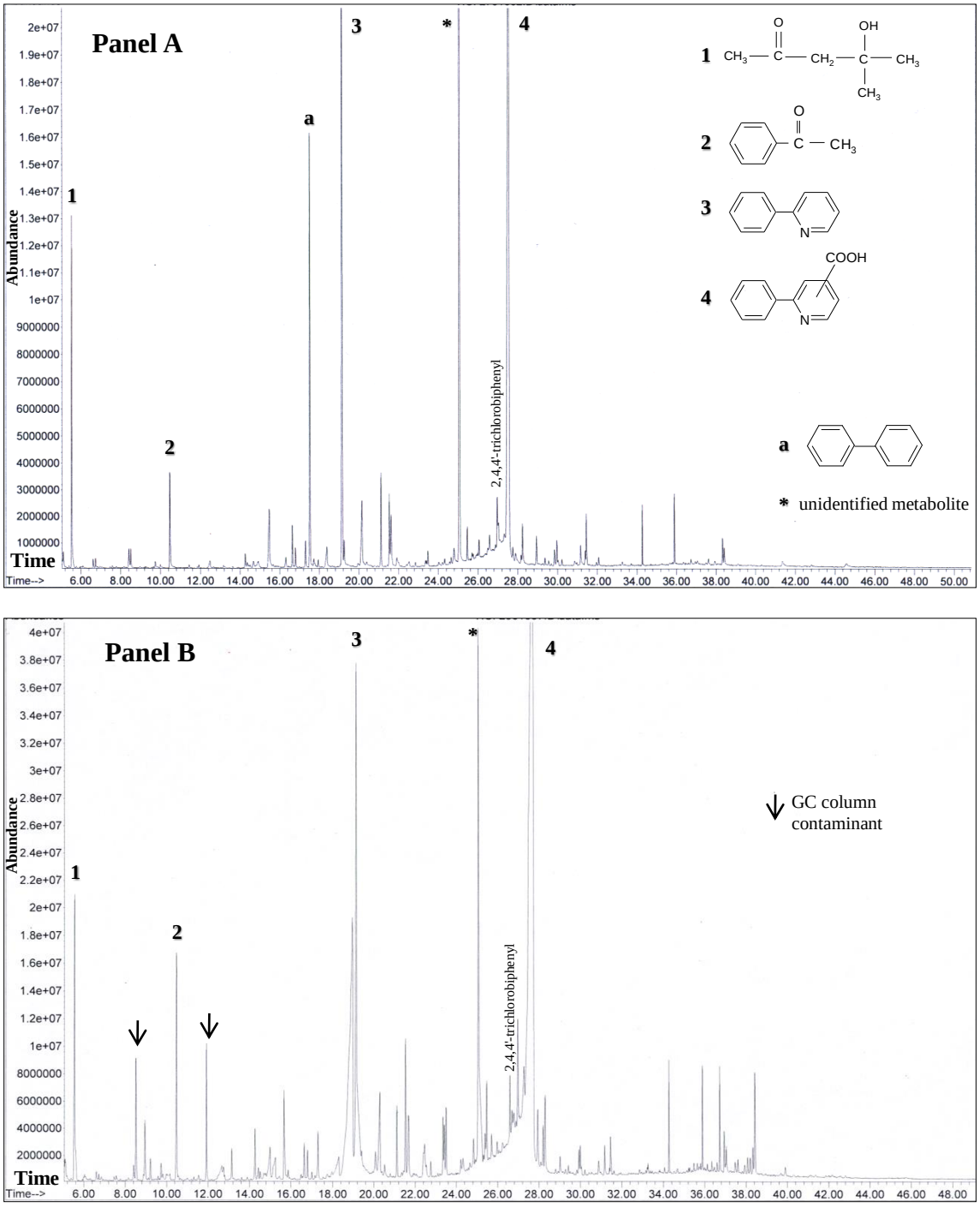


Fig. 1.



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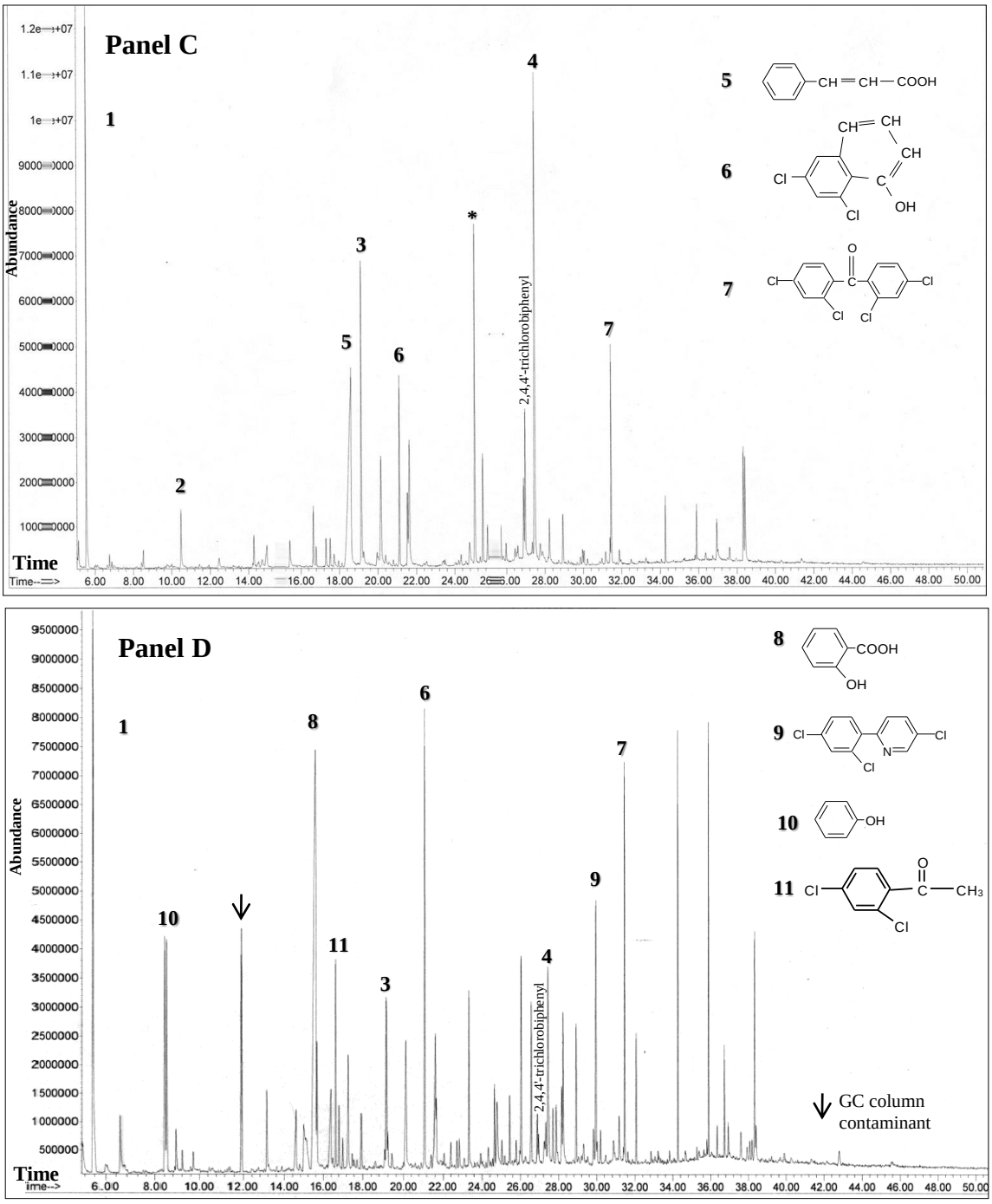
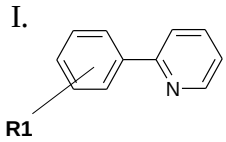
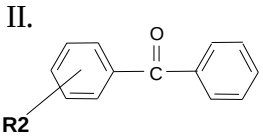
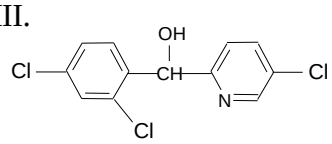

Panel C
Abundance
Time
1
2
3
4
5
6
7
*
2,4,4'-trichlorobiphenyl
5
6
7
Panel D
Abundance
Time
1
10
11
8
6
3
4
2,4,4'-trichlorobiphenyl
9
7
8
9
10
11
GC column contaminant

Fig. 2B.



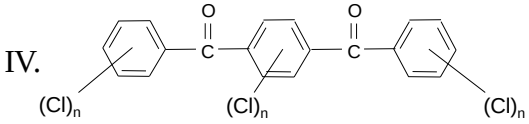
R1= none (*m/z* 77,127,155)
OH (*m/z* 77,115,143,170)
$Cl_{2\ or\ 3}$ (*m/z* 188,153,223; 145,173,250,285)
COOH (*m/z* 77,127,154,199)





R2 = $Cl_{3\ or\ 4}$ (*m/z* 139,145,173,284; 145,173,285,320)

m/z 114,175,252,287



m/z 145,173,277,424; m/z 145,173,311,458

Fig 3.

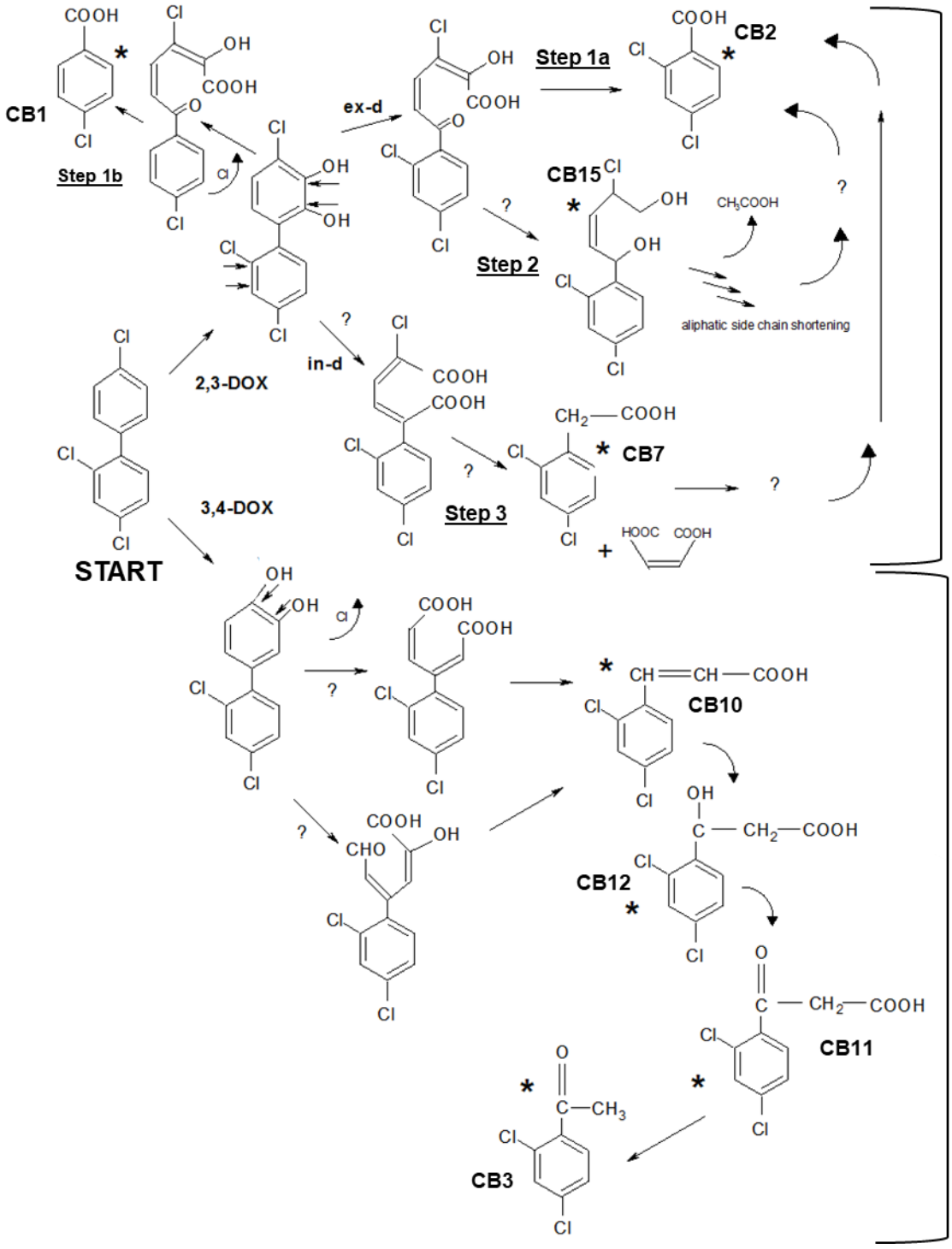


Fig. 4.

Figure captions

Fig. 1. The principal steps in *bph pathway* involved in PCBs microbial transformation and enzymes involved in the upper pathway: BphA, biphenyl-2,3-dioxygenase; BphB, biphenyl-2,3-dihydrodiol-2,3-dehydrogenase; BphC, 2,3-dihydroxybiphenyl-1,2-dioxygenase; BphD (2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase) (Xiang et al., 2020)

Fig. 2A. Full-scan GC-MS chromatograms of the culture extracts after 7-day of incubation of Rhodococcus sp. Z6 (panel A) and Rhodococcus sp. T6 (panel B) with 2,4,4'-trichlorobiphenyl in the presence of biphenyl.

Fig. 2B. Full-scan GC-MS chromatograms of the culture extracts after 7-day incubation of *Rhodococcus erythropolis* R2 (Panel C) and *Rhodococcus ruber* Z57 (Panel D) with 2,4,4‘-trichlorobiphenyl in the presence of biphenyl.

Fig 3. Identification and characteristic *m/z* ions of TPs containing pyridine moieties and those having two or more phenyl groups detected in the culture extracts of rhodococcal strains Z6, T6, R2 and Z57.

Fig. 4. Tentative pathways proposed to be active in rhodococci during biotransformation of congener 2,4,4`-CB (START):

(1) 2,3-dioxygenation (2,3-DOX) of the biphenyl ring (upper part of the scheme)

- *regular bph pathway* leading to the production of 2,4-dichlorobenzoic acid (CB2) **(Step 1a)** and 4-chlorobenzoic acid (CB1) (**Step 1b).**
- *alternative patway* working *via* shortening of the HOPDA aliphatic side chain (**Step 2**) with 5–(2,4-dichlorophenyl)-5-hydroxy-2-chloropentenol (CB15) as one of the TP, leading to the production of 2,4-dichlorobenzoic acid (CB2).
- *alternative pathway* working via intradiol opening of the biphenyl ring (**Step 3**) with 2,4-dichlororphenylacetic acid (CB7) as one of the TP, leading to the production of 2,4-dichlorobenzoic acid (CB2).

(2) 3,4-dioxygenation (3,4-DOX) of the biphenyl ring (lower part of the scheme)

- *alternative pathway* leading to the production of 2,4-dichlororacetophenone (CB3) with dichlorinated cinnamic acid (CB10), 3-(2,4-dichlorophenyl)-3-oxo-propanoic acid (CB11) and 1-hydroxy-1-(2,4-dichlorophenyl)-2-propanoic acid (CB12) as TPs.

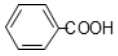
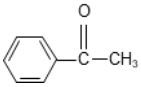
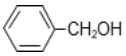
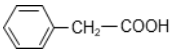
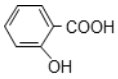
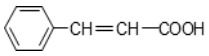
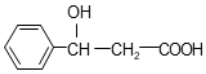
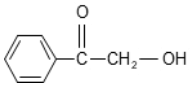
* TPs identifed in the assay with 2,4,4`-CB with details provided in Table 2B
in-d = intradiol opening
ex-d = extradiol opening

Table 1. Information on the PCB degrading *Rhodococcus* strains used in the study.

Label	16S rRNA gene identity	Enriched from the mixed culture	Mixed culture origin [PCB mass fraction in sample (mg kg^{-1})][a]	Sampling location
Z6	*Rhodococcus* sp.[b]	TSZ7	Transformer station soil [6044]	Zadar, Croatia
T6	*Rhodococcus* sp.[b]	AIR1	Airport soil [2.210)	Trogir, Croatia
R2	*Rhodococcus erythropolis*[c]	RMC2	Harbour marine sediment [0.495]	Rijeka, Croatia
Z57	*Rhodococcus ruber*[c]	ZMC57	Harbour marine sediment [0.540]	Zadar, Croatia

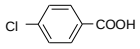
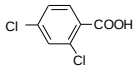
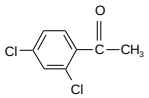
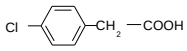
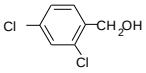
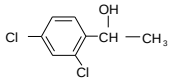
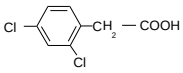
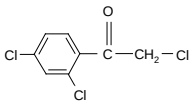
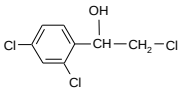
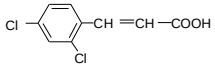
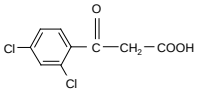
[a] PCB mass fraction in sample (mg kg^{-1}) determined against Aroclor 1248+1254 standards
[b] Petrić et al., 2007
[c] Begonja Kolar et al., 2007

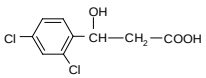
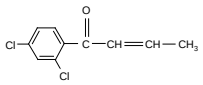
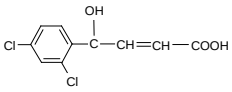
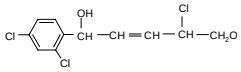
Table 2A. Identification and mass spectra of the TPs formed from biphenyl during 2,4,4'-trichlorobiphenyl biodegradation in cultures of *Rhodococcus* Z6, T6, R2 or Z57.

Transformation product (TP)	TP formula		TP *m/z*[a]	Microbial culture
B1	Benzoic acid		122,105,77,51	Z6, T6, R2
B2	Acetophenone		120,105,77,51	Z6, T6, R2
B3	Phenol		94,66,50	all
B4	Benzyl alcohol		108,91,79,51	all
B5	2-phenylacetic acid		39,65,91,136	Z6, Z57
B6	Salicylic acid		64,92,120,138	Z6, R2, Z57
B7	Cinnamic acid		147,130,103,77	Z6, T6, R2
B8	3-hydroxy-3-phenylpropanoic acid		166,107,79,51	Z6, T6, R2
B9	2-hydroxy-1-phenyl-ethanone		51,77,105,136	Z6

[a] Main mass spectra *m/z* ions

Table 2B. Identification and mass spectra of the chlorinated TPs formed from 2,4,4'-trichlorobiphenyl during biodegradation in cultures of Rhodococcus Z6, T6, R2 and Z57.

Transformation product (TP)	TP formula		TP *m/z*[a]	Microbial culture
CB1	**4-chlorobenzoic acid**		50,75,111,139,156	Z6, Z57
CB2	**2,4-dichlorobenzoic acid**		74,109,145,173,190	all
CB3	**2,4-dichloroacetophenone**		75,109,145,173	all
CB4 2C[b]	4-chlorophenylacetic acid		44,92,170,125,44	Z57
CB5 2C[b]	2,4-dichlorophenyl methanol		51,77,111,141,176	Z6, R2
CB6 2C[b]	α-methyl-2,4-dichlorophenyl methanol		43, 75,111,147,175	Z6, Z57
CB7 2C[b]	2,4-dichlorophenylacetic acid		63,89,125,159,204	all
CB8 2C[b]	2-chloro-1-(2,4-dichlorophenyl)ethanone		75,109,145,173	Z6, T6
CB9 2C[b]	2,4-dichloro-α-(chloromethyl)benzenemethanol		50,75,111,147,175	Z6, R2
CB10 3C[b]	3-(2,4-dichlorophenyl)-2-propenoic acid		74,99,125,136,181,216	all
CB11 3C[b]	3-(2,4-dichlorophenyl)-3-oxo-propanoic acid		232,216,173,145	R2, Z57

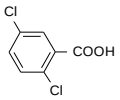
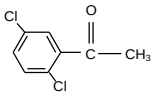
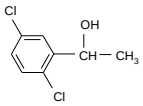
CB12 3C[b]	1-hydroxy-1-(2,4-dichlorophenyl)-2-propanoic acid		232,214,198,175	R2
CB13 4C[b]	1-(2,4-dichlorophenyl)2-butene-1-on		111,173,145	Z6, T6
CB14 4C[b]	4-(2,4-dichlorophenyl)-4-hydroxy-2-butenoic acid		230,195,175,56	Z57
CB15 5C[b]	5-(2,4-dichlorophenyl)-5-hydroxy-2-chloropentenol		282,252,188,175	R2, Z57

[a] Main mass spectra *m/z* ions

[b] Number of C in the aliphatic side chain

Table 3. Identification and mass spectra of chlorinated TPs formed from 2,2',5,5'-tetrachlorobiphenyl during biodegradation in cultures of Rhodococcus Z6, T6, R2 and Z57.

***** Metabolites marked as B1, B2, B4 and B7 presented in the Table 2 were also identified in culture extracts of isolates Z6, T6 and R2. In addition metabolite B6 was identified in the R2 isolate culture extracts.

Transformation product (TP)	**TP formula**		**TP *m/z*[a]**	**Microbial culture**
CB16	**2,5-dichlorobenzoic acid**		74,109,145,173,190	Z6, T6, R2
CB17	**2,5-dichloroacetophenone**		71,145,175,188	all
CB18 2C[b]	α-methyl-2,5-dichlorobenzyl alcohol		111,147,175,190	all

[a] Main mass spectra *m/z* ions

[b] Number of C in the aliphatic side chain