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Biodegradation of trichlorobiphenyls and their hydroxylated derivatives by *Rhodococcus*-strains

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ABSTRACT

A possibility of using a complex approach is considered to explain features of biodestruction of polychlorinated biphenyls (PCBs), which are known to be persistent organic pollutants. The approach comprises the following main stages: *(i)* chemical modification of chloroarenes by hydroxylation and *(ii)* bacterial degradation of the hydroxylated derivatives. This approach is applicable to individual trichlorobiphenyls (PCB 29, PCB 30) and to a widespread mixture Trikhlorbifenil (analog of Aroclor 1242 and Delor 103). As bacterial strain destructors, the *Rhodococcus*-strains (KT112–7, CH628, P25) were used. It was established that the main metabolites of microbial biodegradation of both polychlorobiphenyls and their hydroxy derivatives are polychloro- and hydroxy(polychloro)benzoic acids, which allows an assumption to be made about possible further biodegradation of these compounds down to the products of the base exchange reaction in a cell: water, carbon dioxide and chlorine compounds. The study discusses the effect that the structure of PCBs congeners causes on the conversion by hydroxylation, on the biodegradation rate of both PCBs and their hydroxy derivatives, and on the metabolite formation levels.

1. Introduction

Polychlorinated biphenyls (PCBs), the overall destruction of which by 2028 has been decided upon by the Stockholm Convention, continue to be a factor of high hazard to the humans and to the natural environment. Notwithstanding the ban on production and use of PCBs, the probability of their negative impact on the environmental state of the planet remains rather high, because a considerable amount of these persistent organic pollutants (POPs) was, intentionally or not, transported into the environment and PCBs stay there for the reason of their persistence. Owing to their transborder transfer via air, water and food chains, these chloroaromatic compounds are now revealed in such locations around the globe, where there has been no production using PCBs ever (Bartlett et al., 2019; Nadal et al., 2015; Teran et al., 2012).

PCBs leaking into the environment cause an acute detriment to the state of human health and to the equilibrium of biotas formed, because these toxic compounds are characterized with great half-life periods and can therefore contaminate sites for a rather long time (Sinkkonen and Paasivirta, 2000). For example, for 2,3,4,5,2′,4′,5′-heptachlorobiphenyl (PCB 180) found in the area surrounding the Baltic Sea, the half-life period calculated at 7 °C amounts to 12 thousand hours in air, 240 thousand hours in water, 330 thousand hours in soil, and 333 thousand hours in bottom sediments. The periods are shorter for lower chlorinated PCBs congeners (Sinkkonen and Paasivirta, 2000). Metabolic processes running in the human organism contribute to lower half-life periods of PCBs congeners (Broding et al., 2007; Bu et al., 2015; Hopf et al., 2013; Wimmerová et al., 2011). However, the periods that long are enough to cause a number of serious conditions (Chen et al., 1985; Min et al., 2014).

Research works on the structural transformations of PCBs under the effect of natural factors demonstrate that in the environment these chloroarenes may be subjected to a variety of oxidation processes, including hydroxylation, thus forming hydroxy derivatives (PCBs-OH) (Tehrani and Van Aken, 2014). Recently, sewage water analysis has revealed the presence of PCBs methoxy derivatives (PCBs-OMe) along with the PCBs-OH (Sun et al., 2016, 2018). It has been established that these two classes of compounds are initially formed from PCBs as a result of biotransformation under the action of bacterial strains, and over the

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time of their turnover in nature there can be the interconversion of PCBs-OH and PCBs-OMe, which is evidenced by the on-going changes in their levels in the environment. A suggested mechanism underlying the PCBs-OH – PCBs-OMe interconversion under the action of *Bacillus subtilis* strain is given in detail in the paper (Sun et al., 2018). Unlike PCBs, the PCBs-OH and PCBs-OMe derivatives possess low volatility, which may possibly contribute into their being contained in the soil, water and sediments for longer periods (Sun et al., 2016). At the same time, PCBs-OH and PCBs-OMe, possessing better hydrophilic properties than PCBs, are more available for microbiological degrading.

Earlier, it was established that the strain of *Rhodococcus wratislaviensis* KT112–7 (Egorova et al., 2019) is an efficient strain destructor for the mixture of PCBs-OH, PCBs-OMe derivatives (hydroxymethoxy)polychlorobiphenyls (PCBs-OH,OMe) synthesized via the interaction between the Russian-made technical PCBs mixture Sovol (analog of Aroclor 1254) (Gorbunova et al., 2014) and sodium methoxide in the medium of DMS/MeOH (Plotnikova et al., 2017). It was demonstrated that the biodegradability of the mixture of PCBs-OH, PCBs-OMe, PCBs-OH,OMe derivatives amounted to 73 – 100% depending on the initial concentration in the range of 0.10 – 1.50 g/l. The most optimal and stable result was achieved for the bacterial destruction of the mixture of PCBs derivatives at a concentration of 0.10 g/l. Substituted catechol and benzoic acids were found to be intermediate compounds, which can also be utilized in the process of biodegradation using the strain *Rhodococcus wratislaviensis* KT112–7.

The bacterial strain of the same genus of *Rhodococcus ruber* P25 in consortium with the strain *Microbacterium* sp. B51 is applied successfully for bioremediation of soil contaminated with the PCBs mixture Sovol (Egorova et al., 2013a). Bi- and trinomial consortia of the strains *Rhodococcus ruber*, *Achromobacter xylosoxidans*, *Stenotrophomonas maltophilia* and *Ochrobactrum anthropi* are found to be effective for bio-purification of soil containing as a contaminant the mixture PCBs Delor 103 commercially produced in Czechoslovakia (analog to the Russian-made Trikhlorbifenil and to the American Aroclor 1242) (Horváthová et al., 2018).

It is relevant to add that some research works on the biodegradation of PCBs contain data indicating a greater toxicity of PCBs-OH as compared to the initial compounds, which is based on the studies of metabolic pathways of PCBs transformation in a living organism and on the effect that the metabolic products cause on certain organs and their functions (Meerts et al., 2002; Mizukami-Murata et al., 2016). It should be noted that, unlike for the exposure to PCBs, there are no quantitative standards developed for the levels of PCBs-OH because the toxicity of PCBs-OH depends on the number of chlorine atoms and HO-groups in the structure of biphenyl and also on their positions in two aromatic cycles (Meerts et al., 2002); and there are very few data available on the toxicity of individual compounds of PCBs-OH. Further studies are necessary to explore the destruction of PCBs and their metabolites under the action of bacterial strains. The most promising of the studies appear to be the research works covering the use of aerobic strains, because approximately 40% of the PCBs ever produced are present in the environment (Zanaveskin and Averyanov, 1998).

As a rule, to study PCBs metabolites, the bacterial strains are used that are adapted to the PCBs themselves. The purpose of the present research is to study biodegradation of trichlorinated congeners and the PCBs-OH produced thereof under the action of aerobic bacterial strains *Rhodococcus wratislaviensis* KT112–7 (KT112–7), *Rhodococcus wratislaviensis* CH628 (CH628) and *Rhodococcus ruber* P25 (P25). The choice of trichlorinated biphenyls and their hydroxylated derivatives are based on the prevailing presence of trichlorinated congeners in the technical PCBs mixture known under the name Trikhlorbifenil and widely spread in Russia. So, the study of biodestruction of the mixture of PCBs-OH synthesized from the commercial product Trikhlorbifenil appears to be a logical contribution to the present research.

In this study, the compounds and mixtures synthesized under laboratory conditions were used as model substances to obtain the results to be used for outlining the main directions of bioutilization of polychloro (hydroxy)arenes and to be proposed as a tool for interpreting the real processes occurring in the environment.

2. Materials and methods

2.1. Making mixture Trikhlorbifenil

The Russian-made product Trikhlorbifenil is a mixture of over 50 PCBs congeners and is composed of di- (14.5%), tri- (47.7%), tetra- (29.3%) and pentachlorobiphenyls (3.8%) (Pervova et al., 2015). Quantitatively and qualitatively, its composition corresponds to the PCBs mixture Aroclor 1242 (USA) (Fischer and Ballschmiter, 1989; Frame, 1997; Hillery et al., 1997).

2.2. Reagents and chemicals

The following reagent were used for the synthesis process: benzene, 2,4,5- and 2,4,6-trichloroanilines, 2-aminoethanol, potassium hydroxide, *iso*-amyl alcohol, sodium nitrite, hydrochloric acid and toluene. All the reagents were of CP grade (Russia).

The following reagents were used for analytical studies:

1. mono- (2-, 3-, 4-), di- (2,4-, 2,6-, 3,5-, 3,4-, 2,3-, 2,5-), tri- (2,4,6-, 2,4,5-, 2,3,6-) and tetra- (2,3,4,5-) chlorobenzoic acids from Sigma-Aldrich (Germany);
2. mono- (3-, 4-) and dihydroxy- (3,4-) benzoic acids from Sigma-Aldrich (Germany);
3. 2-hydroxy-3-chloro- and 2-hydroxy-4-chlorobenzoic acids from Sigma-Aldrich (Germany);
4. unsubstituted biphenyl (99% purity) from Acros-organics (USA);
5. mineral salts and acids (> 98% purity) from ZAO Ekros (Russia);
6. and solvents for HPLC (HPLC grade) from Kriokhrom (Russia).

2.3. Synthesis of trichlorinated congeners

To study biotransformation of trichlorobiphenyls and to synthesize from them the PCBs-OH by Gomberg-Bachmann-Hey reaction in the presence of *iso*-amyl nitrite (Mullin et al., 1984), two trichlorinated PCBs congeners were synthesized: 2,4,5-trichlorobiphenyl (PCB 29) was obtained from 2,4,5-trichloroaniline and benzene; and 2,4,6-trichlorobiphenyl (PCB 30) was produced from 2,4,6-trichloroaniline and benzene.

2.4. Synthesis of hydroxy derivatives

The same synthesis procedure was followed to obtain PCBs-OH from PCB 29, PCB 30 and commercial PCBs mixture Triklorbifenil. In a flask equipped with a magnetic mixer, a reflux condenser and a drop funnel, 3.36 g (0.06 mol) of KOH and 25 ml (25.55 g, 0.42 mol) of 2-aminoethanol were added. Under intense stirring, the flask's content was heated up until fine-dispersed suspension was formed. Then 2.56 g (0.01 mol) of PCB 29 or PCB 30 or PCBs mixture Triklorbifenil were added, and the reaction mass was boiled under stirring at ~170 °C for 13 hrs. Upon completion, the reaction mass was cooled down to room temperature, and diluted hydrochloric acid was added to a pH of 1–2, and then extraction with 2 × 10 ml of toluene was carried out. The toluene extract was dried with $CaCl_2$ and then analyzed under the GC/MS conditions.

Each interaction was carried out three times. For the purpose of microbiological studies, toluene extract was evaporated in air and the residue was degassed (5 mm Hg, room temperature) until constant mass was achieved.

2.5. Analysis of initial compounds and interaction products

The initial compounds and interaction products were identified and their compositions determined using an Agilent GC 7890 A MS 5975 C

Inert XLEI/CI gas chromatograph mass spectrometer (USA) (GC/MS conditions) equipped with an HP-5MS quartz capillary column (polydimethylsiloxane, 5 mass% of phenyl groups) of 30 m in length, 0.25 mm in diameter, with a film thickness of 0.25 μm, and equipped with a quadrupole mass spectrometer detector. Temperature of the column: initial temperature, 40 °C (3 min hold); oven heating programmed at a rate of 10 grad/min up to 290 °C (30 min hold); evaporator temperature, 250 °C; ion source temperature, 230 °C; quadrupole temperature, 150 °C; intermediate chamber temperature, 280 °C; helium as a carrier gas, 1.0 ml/min. Scanning in the Electron Ionization mode (70 eV) with a total ion current (TIC) across the mass range of 20 − 1000 amu.

The work was carried out using the NIST2014 database. Quantitative assessment of the relative contents of the interaction products was carried out following the method of internal normalization according to the peak areas in the chromatograms relative to the total peaks area of all the compounds recorded.

GC analysis of each reaction products was also performed three times. The standard deviation of measurements did not exceed 4%.

2.6. Bacterial strains and culture conditions

The *Rhodococcus*–strains used in the present study were isolated by the method of enrichment cultivation from soils contaminated with chloroaromatic compounds taken from different climatic zones. An enrichment cultures were obtained by the incubation of 1 g soil sample in 100 ml of the mineral medium (Raymond's or K1 medium) (Plotnikova et al., 2006; Egorova et al., 2013b, 2017a) which contains biphenyl or *ortho*-phthalic acid (1 g/l) as selective factor. Individual strain-destructors were obtained from the enrichment culture by plating on an agarized K1 medium with biphenyl.

The *Rhodococcus*–strains used in the present study were isolated from soils contaminated with chloroaromatic compounds taken from different climatic zones. The strain *Rhodococcus ruber* P25 was isolated from the soils of Serpukhov (Moscow region, Russia) and was found capable of degrading a wide spectrum of chloroaromatic compounds, including PCBs (Plotnikova et al., 2006, 2012). The strain *Rhodococcus wratislaviensis* CH628 was extracted from the soils of Chapayevsk (Saratov region, Russia) contaminated with PCBs and was found capable of degrading hexachlorocyclohexane and 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) (Egorova et al., 2017a, 2017b). The strain *Rhodococcus wratislaviensis* KT112–7 was extracted from the soils of Berezniki (Perm Territory, Russia) contaminated with aromatic compounds and was found to degrade chloroaromatic compounds including PCBs under various salinity conditions (Egorova et al., 2013b, 2018). The cultures were sustained in the active condition by using the periodic cultivation method in liquid mineral medium K1 (Zaitsev et al., 1991) with the introduction of biphenyl (1 g/l) as a source of carbon using a BioSan ES-20/60 orbital shaker (Latvia) at 120 rpm and at a temperature of + 28 °C.

2.7. Biodegradation experiments

Microbiological experiments were carried out using bacterial cultures pre-grown in K1 mineral medium (composition (g/l): K_2HPO_4•3 H_2O – 3.2, NaH_2PO_4•2 H_2O – 0.4, $(NH_4)_2SO_4$ – 0.5, $MgSO_4$•7 H_2O – 0.15, $Ca(NO_3)_2$ – 0.01) (Zaitsev et al., 1991) containing biphenyl at a concentration of 1 g/l as an additional carbon source, until the middle exponential phase (OD_{600} = 1.5). The 1 ml (0.8* 10^4 CFU/ml) of incubated bacterial strains were placed in 4 ml Wheaton sample vials (Sigma-Aldrich, Germany) closed with PTFE-lines stopper. The investigated chemical compounds were added at a final concentration of 0.1 g/l (1 μl of concentrated acetone solution / 1 ml of cell culture) and incubated on a shaker BioSan ES-20/60 (Latvia) at 120 rpm, + 28 °C for a given time. Samples for analysis were taken immediately upon mixing of the bacterial cultures with the compounds under study and on 0, 1, 3, 7, 10 and 14 days of incubation. Each time five samples were selected. The biodestruction process was stopped by freezing.

The following model conditions were created for the purpose of control: heat-killed bacterial cells were placed in vials and solution of the corresponding test substrates was added. Samples were taken for analysis immediately after addition and on the 14th day of incubation. There was also cell-free control. In this case, the vials contained the K1 mineral medium and substrate. The analysis of the control samples showed that there was no change in substrate concentration (the difference in the values of control samples fitted into the confidence interval when statistically calculated). Possible metabolites were not identified in the control samples.

Since changes in the concentration of substances in the control samples were not recorded, these data are not included in the figures so as not to clutter up the space of the figures.

Bacterial growth was monitored in unfrozen samples by the optical density at 600 nm on the spectrophotometer BioSpec-mini (Shimadzu, Japan). To control the growth of bacterial cultures, we used the bacterial biomass under the conditions of biodegradation with no PCBs / PCBs-OH introduced in the medium.

2.8. Analysis after biodegradation

To perform analysis by gas chromatography with a flame ionization detector (GC/FID), in a flask containing water medium K1 with bacterial cells, 0.1 ml of concentrated HCl was added and extraction was carried out using hexane at a ratio of water to organic phase 2:1. For better phase separation, the flasks with extract were subjected to centrifuging for 5 min using a Sigma 3–16 P (Sigma, Germany) centrifuge at a rate of 10,000 rpm. Then an organic layer was analyzed.

Quantitative assessment was carried out in the GC/FID conditions: a Shimadzu GC 2010 gas chromatograph equipped with a flame ionization detector and a ZB-5 capillary GC silica column (Shimadzu, Japan) with the dimensions of 30 m x 0.25 mm and a film thickness of 0.25 μm. The initial column temperature was 40 °C (3 min) followed by temperature increase at a rate of 10°/min up to the finial temperature of 280 °C (isotherm for 15 min). The evaporator temperature was 250 °C, and the detector temperature was 300 °C.

The calculation of PCBs-OH content in every sample was performed by internal normalization to calculate contributions by each compound into the total peak area. Based on the calculated peak areas, the contents of PCBs-OH were estimated after the biodegradation process.

2.9. Analysis of metabolites

The metabolites were investigated in the samples taken on 0, 1, 3, 7, 10 and 14 days of the biodegradation experiment. The content of each vial was centrifuged using a MiniSpin centrifuge (Eppendorf, Germany) for 10 min at 12000 rpm. Supernatant was used for the analysis.

Accumulation of metabolites (polychlorobenzoic and hydroxy(polychloro)benzoic acids) in the culture medium was detected by HPLC method using a LC-20 CE Prominence chromatograph (Shimadzu, Japan) equipped with a column (C-18 150 ×4.6 mm; Sigma-Aldrich, USA) and a SPD-20A UV detector (at 205 nm) in the acetonitrile – 0.1% H_3PO_4 (60:40) system. The metabolic products were identified comparing the time of holding the formed products and the reference compounds (Maltseva et al., 1999).

It is established that mono-, di-, tri- and tetrachlorobenzoic acids in the selected HPLC conditions are recorded at a wavelength (λ) of 232 nm in the hold range (τ) of 5÷11 min; mono- and dihydroxybenzoic acids at λ = 226 nm and τ = 4÷6 min; and mono(chloro/hydroxy)benzoic acids, at λ = 205 nm and τ = 10÷15 min

The formation of (chloro/hydroxy)− 2-hydroxy-6-oxo-6-chlorophenyl-2,4- hexadienoic acids (HOPDA) was determined using a UV-Visible BioSpec-mini spectrophotometer (Shimadzu, Japan) at λ_{max} ranging from 390 to 440 nm (Maltseva et al., 1999). Accumulation of chloride ions in the medium during biodegradation was monitored by

measuring the optical density of silver chloride using a UV-Visible Bio-Spec-mini (Shimadzu, Japan) at λ_{460}, formed after the reaction of chloride ion with silver nitride. Concentrations were calculated according to the calibration curve. The calculation of amount of chloride ions detected in the medium as a percentage of the maximum possible was made according to the formula:

$$C_{\%} = C_{exp} \cdot 100\% / C_{max} \quad (1)$$

where $C_{\%}$ – concentration of isolated chloride ion from the maximum possible, %; C_{exp} – concentration of isolated chloride ion in the medium at each experimental point, mg/l; C_{max} – amount of chloride ion contained in 0.1 mg/l of the mixture M3.

2.10. Statistical analysis

All the experiments were conducted in triplicate. Statistical analysis was carried out using STATISTICA 6.0 and CAKE. The paper presents results with statistically validity.

3. Results and discussion

3.1. Investigation of products obtained by hydroxylation of individual trichlorobiphenyls and of the commercial mixture Triklorbifenil

Because trichlorinated congeners make the major contribution into the composition of the wide-spread Russian product PCBs mixture Trikhlorbifenil (Pervova et al., 2015), the choice was made to use 2,4,5- (PCB 29) and 2,4,6- (PCB 30) trichlorobiphenyls for our investigation.

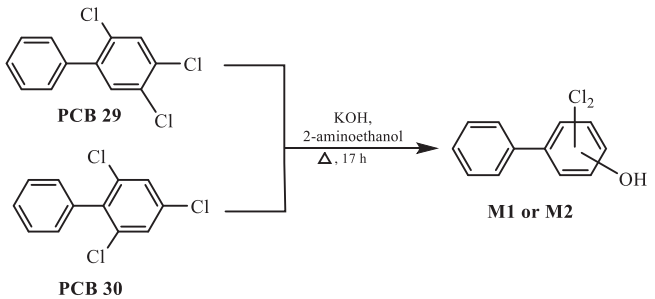
It was shown earlier that, through the interaction between the PCBs mixture Sovol and KOH in the medium of 2-aminoethanol (2-AE) at 170 °C during 13 hrs, a mixture of mono- (64%), di- (29%) and trihydroxy derivatives (< 3%) of PCBs can be obtained with 96% conversion of the initial compounds (Maiorova et al., 2017). The reaction was running following the mechanism of nucleophilic substitution of chlorine atoms by HO-groups.

Similar method was used to realize hydroxylation of PCB 29 and PCB 30. Using the GC/MS method, it was found that, from PCB 29, a mixture of three hydroxydichlorobiphenyl isomers (mixture M1) was formed; and from PCB 30, a mixture of two hydroxydichlorobiphenyl isomers (mixture M2) was obtained. Both interactions resulted in 100% conversion (Scheme 1).

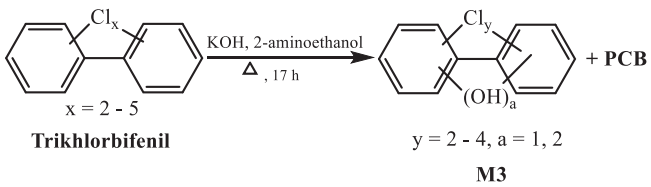
The synthesized isomeric hydroxy derivatives in mixtures M1 and M2 are most likely to be the products of the standard *ipso*-substitution: mixture M1 is composed of 2-hydroxy-4,5-dichloro-, 4-hydroxy-2,5-dichloro- and 3-hydroxy-4,6-dichlorobiphenyls; and mixture M2, of 2-hydroxy-4,6-dichloro- and 4-hydroxy-2,6-dichlorobiphenyls. Mass spectra of the compounds from the mixtures M1 and M2 are given in Supplementary Data (Figs. S1–S4).

Similar procedure was used to treat the commercial PCBs mixture Trikhlorbifenil (its initial congener content is presented in Table S1) and mixture M3 was obtained yielding 79.5% conversion (Scheme 2).

Using the GC/MS method, it was found that mixture M3 is composed



Scheme 1. Hydroxylation of PCB 29 and PCB 30.



Scheme 2. Hydroxylation of mixture Trikhlorbifenil.

of di- and trichlorobiphenyls (PCBs), which in the chemical reaction conditions remained unchanged, and of hydroxy derivatives (PCBs-OH). The data on the types and the quantitative contributions of M3 mixture components are given in Table 1. Typical mass spectra of the compounds of the mixture M3 are given in Supplementary Data (Figs. S5 and S6).

Analysis of Table 1 demonstrates that in the interaction conditions according to Scheme 2, of all the trichlorobiphenyls contained in the mixture Trikhlorbifenil (47.7%), approximately five sixths of them underwent chemical transformation. Evidently, the obtained result depends to a great extent on the location of chlorine atoms in the biphenyl structure. The congeners involved in the interaction according to Scheme 1 exhibited the greatest reactivity.

The PCB 29 and PCB 30 congeners have the structures that show as similarities as some differences. The both compounds are characterized with the presence of all chlorine atoms associated in one aromatic ring only, with the second ring of the biphenyl structure unsubstituted. However, the different locations of chlorine atoms in PCB 29 and PCB 30 are responsible for the different physical and chemical properties exhibited by the congeners. For example, calculations of dihedral angles (φ) between the benzoic rings revolving around a simple C-C bond, which were performed in the framework of the DFT theory using a hybrid potential RB3LYP\6–31 G**, demonstrate that this angle for PCB 29 amounts to 53°, while for PCB 30 it equals to 90° (Bureš et al., 2008). The angle φ for PCB 29 is comparatively small, which is evidence of a greater delocalization of the electron density between the two aromatic rings in this congener as compared to PCB 30. That defines PCB 29 as a congener of higher stability and lower reactivity as compared to the congener PCB 30 (Gorbunova et al., 2014). However, according to the Scheme 1 interaction, both compounds showed 100% conversion.

When analyzing the congener composition of PCBs mixture Trikhlorbifenil (Pervova et al., 2015) with due consideration to the data reported in (Gorbunova et al., 2014), the trichlorinated components of this mixture could be divided into two groups: one group comprises the trichlorobiphenyls having the angle φ within the range of 48÷56° (PCB 20, PCB 22, PCB 25, PCB 26, PCB 28, PCB 31, PCB 33, PCB 37); and the second group, with the angle φ ranging as 73÷90° (PCB 16, PCB 17, PCB 18, PCB 19, PCB 24, PCB 27, PCB 32). Quantitatively, the cumulative contribution by the first group members is over 28%, and by the second group members, approximately 20%. It should also be noted that among the specified fifteen trichlorobiphenyls, only PCB 24 has the structure similar to that of PCB 29 and PCB 30, where all the chlorine atoms are positioned in one of the aromatic rings. The other trichlorobiphenyls contained in the PCBs mixture Trikhlorbifenil have chlorine atoms distributed over the both rings of the biphenyl structure as {2 + 1}. It could be assumed that the lack of complete conversion in the interaction between the mixture Trikhlorbifenil and KOH in the presence of 2-AE

Table 1
Relative quantitative assessment of the compounds' contents in mixture M3.

Compound	Molecular mass, amu	Content, %[1]
Dichlorobiphenyls ($C_{12}H_8^{35}Cl_2$)	222	12.8
Trichlorobiphenyls ($C_{12}H_7^{35}Cl_3$)	256	7.7
Hydroxydichlorobiphenyls ($C_{12}H_7^{35}Cl_2OH$)	238	48.6
Hydroxytrichlorobiphenyls ($C_{12}H_6^{35}Cl_3OH$)	272	30.9

[1] calculated following the method of internal normalization over the areas of peaks

(Scheme 2) is partially related to the specific structure of the trichlorinated congeners included in the composition of the initial product and belonging to the first group with the angle φ. As a proof supporting this assumption, there are the results of interaction between the Russian-made PCBs mixture Sovol and a hard nucleophile MeONa, when PCB 22, PCB 28 and PCB 33 ($\varphi = 52 \div 58^{o}$) have trichlorobiphenyls that remain unreacted (Bureš et al., 2008). These trichlorobiphenyls are also contained in the PCBs mixture Trikhlorbifenil.

3.2. Investigation of biodegradation of PCB 29 and PCB 30 congeners and mixtures of hydroxy derivatives M1, M2

Further, the PCB 29 and PCB 30 congeners and the mixtures of hydroxy derivatives M1, M2 were subjected to biodegradation under the action of the bacterial strains KT112–7, CH628 and P25. Biodegradation was evaluated using the GC/FID method; and the metabolites were identified and analyzed by HPLC method.

Analysis of the data on biotransformation of PCB 29, PCB 30 congeners demonstrates that each of the three bacterial strains have provided complete destruction of PCB 30 in 14 days; while in the case of PCB 29, its period of biodegradation varies: in the presence of strain CH628, the period amounts to 10 days; and in the presence of P25, 7 days; in the presence of strain KT-112–7, 3 days. The results are displayed in Fig. 1.

When studying biodegradation of mixture M2, the result obtained was similar to the result for PCB 30: all three strains destruct this product in 14 days. For mixture M1, the biodegradation times are different: in the presence of strains KT-112–7 and P25, it is 10 days in each case, while with the strain CH628, it is 14 days. The results are displayed in Fig. 2.

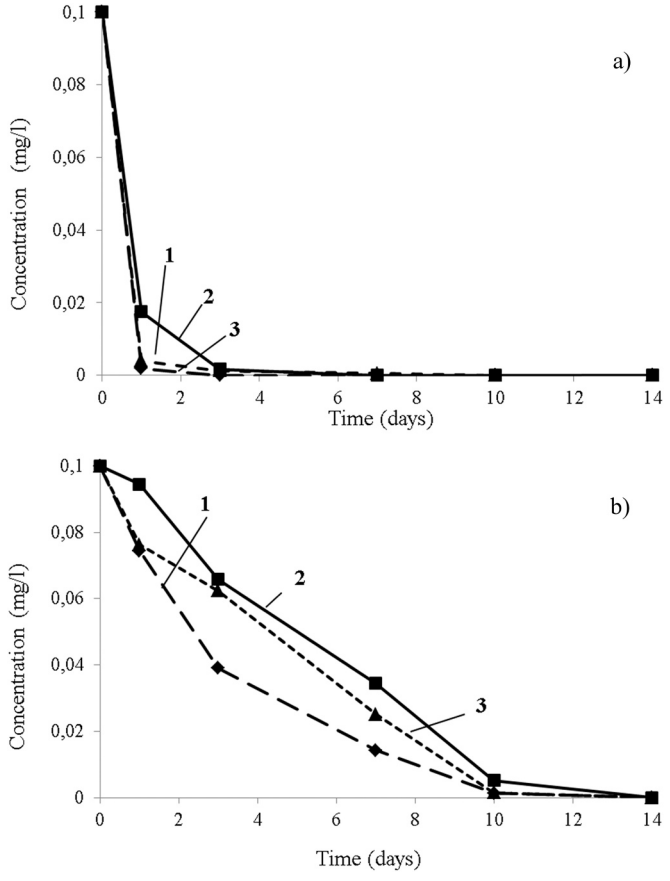


Fig. 1. Concentration as a function of biodegradation time under the action of bacterial strains CH628 (1), KT112–7 (2) and P25 (3) within 0 – 14 days: *a)* for PCB 29; *b)* for PCB 30.

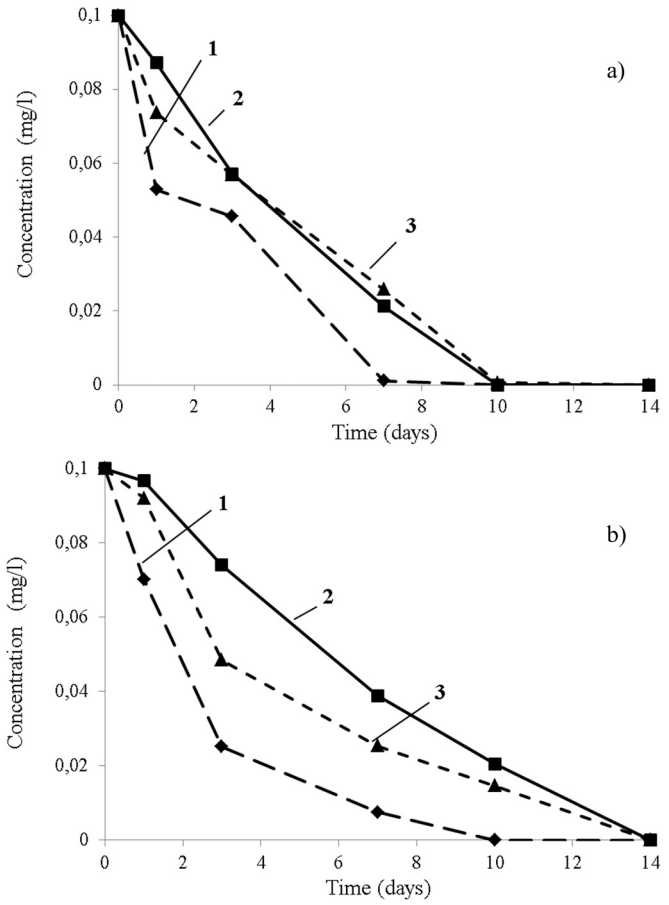
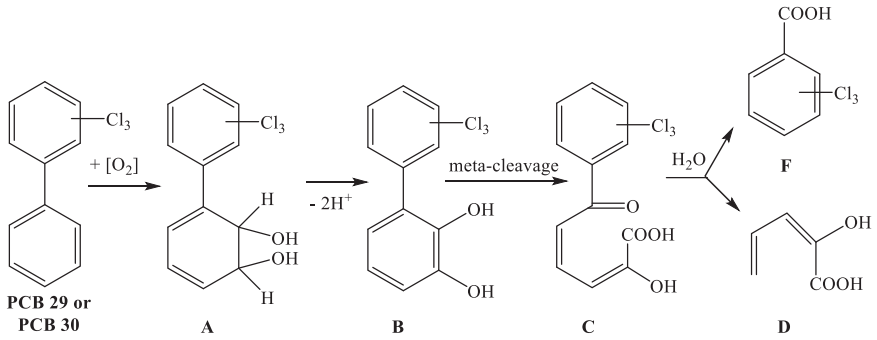


Fig. 2. Concentration as a function of biodegradation time under the action of bacterial strains CH628 (1), KT112–7 (2) and P25 (3) within 0 – 14 days: *a)* for mixture M1; *b)* for mixture M2.

Low-chlorinated (the number of chlorine atoms ≤ 3) PCBs congeners in the presence of aerobic microorganisms are known to be subjected predominantly to oxidation degradation under the effect of enzymes of oxygenase class to form catechol-like compounds (Furukawa and Fujihara, 2008) Apparently, for PCB 29 and PCB 30 congeners, for attacking two oxygen atoms of the oxygen molecule activated by dioxygenase, the most favorable structural fragment is the unsubstituted aromatic ring (Scheme 3). Oxidation of the initial congeners is carried out via dihydroxylation stage to form the structures A, which are further subjected to aromatization showing the splitting of two protons ($2H^{+}$), thus forming diols B. Then a standard for aerobic bacteria *meta*-cleavage of unchlorinated ring occurs, and substituted hexadienoic acids are formed with the structure C (HOPDA).

Further cleavage of HOPDA acids (C structure compounds) is associated with the action by hydrolase enzyme, which results in the formation of pentadienoic D and trichlorobenzoic F acids (Passatore et al., 2014) (Scheme 3). Further, pentadienoic acid D is converted down to the products of the base exchange reaction in a cell using the enzyme systems of the low biphenyl pathway, and acid F is converted into polychlorocatechols provided that the bacterial strains have specific enzyme systems.

After biodegradation of PCB 29 and PCB 30, the HPLC results show the presence of trichlorobenzoic acids F. The revealed trichlorobenzoic acids were identified by comparison between the times of holds with the reference benzoic acids. It was found that in the case of PCB 29, 2,4,5-trichlorobenzoic acid is produced as a metabolic product; and in the case of PCB 30, it is 2,4,6-trichlorobenzoic acid. The obtained data serve to prove that with all three strains the biodegradation of PCB 29 and PCB 30 occurs following Scheme 3. The results are displayed in Fig. 3.



Scheme 3. Probabilistic pathway of oxidation biodegradation of trichlorobiphenyls in the presence of aerobic bacteria (Furukawa and Fuijhara, 2008; Passatore et al., 2014; Tehrani and Van Aken, 2014).

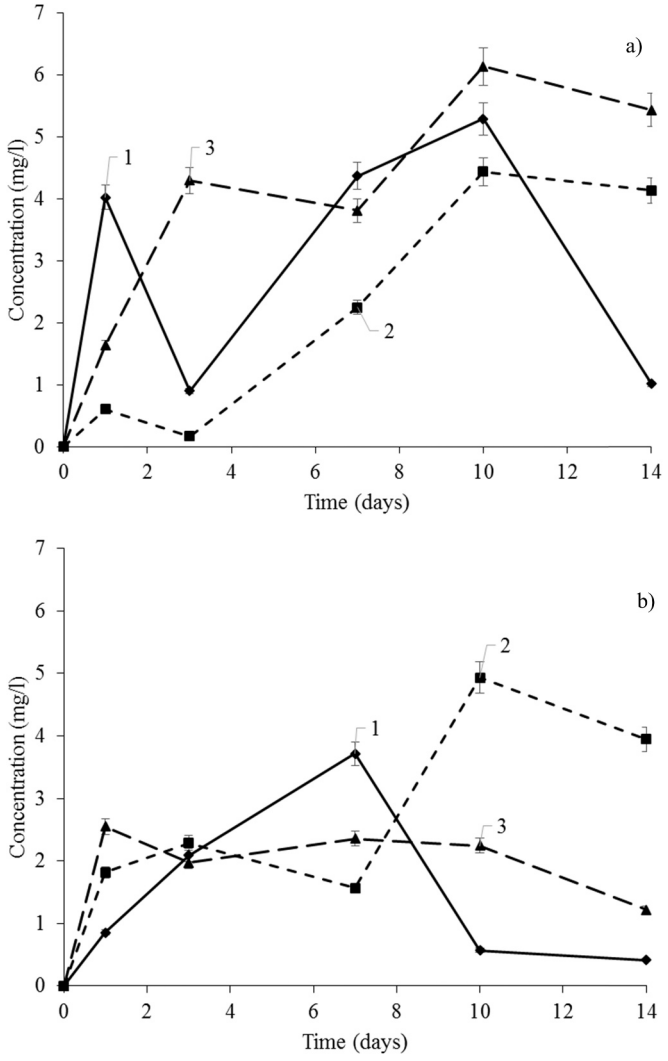


Fig. 3. Concentrations for trichlorobenzoic acids as a function of time of PCB 29 (*a*) and PCB 30 (*b*) biodegradation by the strains of CH628 (1), KT112–7 (2) and P25 (3).

The strains CH628, KT112–7 and P25 are known to possess enzyme systems that allow for the transformation of the chlorinated benzoic acids formed in the process of biodegradation (Plotnikova et al., 2012; Egorova et al., 2013b).

The significant difference found between the times of the PCB 29 and PCB 30 bacterial degradation in the presence of three strains seems to be associated with the chemical structure of both the initial congeners and their metabolites. This statement is supported with the same results obtained on the biodegradation of PCB 30 over the entire scale of the strains and the different biodegradation times observed for PCB 29. In PCB 29 and PCB 30 congeners, the duration of the first stage of dihydroxylation of the unsubstituted ring by enzyme-generated oxygen radical would hardly be different. Rather, the significant differences are related to the following processes of aromatization, *meta*-cleavage and splitting of the substituted pentadienoic acids **C**. In the described biodegradation conditions, the symmetrical arrangement of three chlorine atoms are assumed to provide a greater stability of PCB 30 and its derivatives as compared to PCB 29 and its derivatives. The spectrophotometry results are in favor of this assumption: as PCB 30 (but not PCB 29) is degraded with the strain of P25, temporal culture accumulation of structure **C** in the medium is detected: wavelength is 392 nm, OD = 0.217 – 0.477. Degrading performed with the strains of KT112–7 and CH628 did not show compound **C** accumulation in the medium. However, this assumption requires a separate and in-depth study.

The metabolites of the mixtures M1 and M2 show no fundamental differences from the metabolites of PCB 29 and PCB 30. The HPLC analysis of the mixtures M1 and M2 at a wavelength of 232 nm in the UV range, no peaks were detected characteristic of polychlorobenzoic acids. However, the analysis of both mixtures at a wavelength of 205 nm revealed the peaks corresponding to two substances with the times of holding at 12.6 and 14.1 min. Analysis of the reference compounds demonstrated that this range corresponds to the time of holding for hydroxy(polychloro)benzoic acids. Therefore, it has been established that, upon the action of the bacterial strains, the metabolites are hydroxydichlorobenzoic acids for both mixtures M1 and M2 rather than trichlorobenzoic acids F from Scheme 3 reported for degradation of PCB 29 and PCB 30. The obtained data indicate a similar biodegradation pathway for the mixtures M1 and M2 as compared to the initial PCB 29 and PCB 30 congeners.

In the PCB 29 and PCB 30 hydroxy derivatives, the presence of HO-group instead of chlorine atom resulted from the reaction according to Scheme 1 has led to the time of biodestruction being unchanged for M2 as compared to the time for the initial congener, and the time amounts to the same 14 days; while for mixture M1, the biodegradation period has increased with all the three bacterial strains (Fig. 4).

The greatest increase in the time of degradation of mixture M1 was observed under the action of KT112–7 (from 3 to 10 days). Apparently, the presence of HO-group in PCB 29 hydroxy derivatives, which are not involved in the transformation sequence according to Scheme 3, cause a toxic effect on the bacterial strains (Sondossi et al., 1991; Camara et al., 2004; Yamada et al., 2006; Tehrani et al., 2014; Tehrani and Van Aken, 2014; Bhalla et al., 2016). As for mixture M2, such conclusion is irrelevant so long as PCB 30 and its hydroxy derivatives are characterized with the same time of biodegradation (14 days).

The mixture M1 contains 2-hydroxy-4,5-dichloro-, 4-hydroxy-2,5-dichloro- and 3-hydroxy-4,6-dichlorobiphenyls, and the mixture M2 contains 2-hydroxy-4,6-dichloro- and 4-hydroxy-2,6-dichlorobiphenyls.

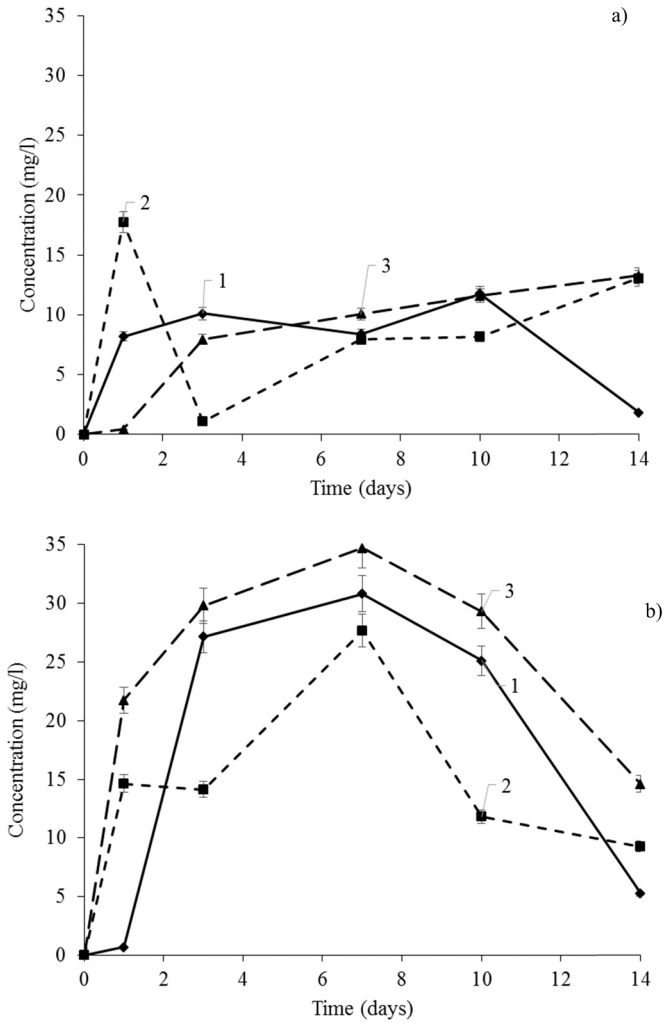


Fig. 4. Concentrations for hydroxydichlorobenzoic acids as a function of time of biodegradation of mixture M1 (*a*) and M2 (*b*) by the strains of CH628 (1), KT112–7 (2) and P25 (3).

Based on the data reported in Bhalla et al. (2016), the presence of the substituents in the positions {2-OH + 5-Cl}, {4-OH + 5-Cl} and {3-OH + 4-Cl} in the biphenyl molecule causes greater toxicity than the presence of the substituents in the positions {2-OH + 4-Cl} and {4-OH + 2-Cl}, regardless of the presence of other substituents. For the first group, EC_{50} varies within 0.34–6.34 mg/l; for the second group, EC_{50} varies within 0.07–0.09 mg/l. Probably, the appearance of HO-group in the position 2 or position 4 in a molecule of PCB 30 upon hydroxylation does not increase the toxicity level of the molecule for the strains presented in the study. That results in the M2 mixture degradation time being kept at a level of the degradation time of PCB 30.

3.3. *Biodegradation of mixture M3 obtained from the commercial product Trikhlorbifenil*

Biodegradation of mixture M3 was also investigated under the action of the three bacterial strains. Fig. 5 displays the total peaks areas for the components of mixture M3 as a function of biodegradation time within 0 – 14 days.

The data from Fig. 5 demonstrate that the strains of KT112–7 and CH628 destruct all the components of M3 mixture completely in 14 days, with the rate of degradation of chloroarenes from mixture M3 being higher with the KT112–7 bacterial strain. On the contrary, in the similar conditions, the bacterial strain P25 shows a lower degrading

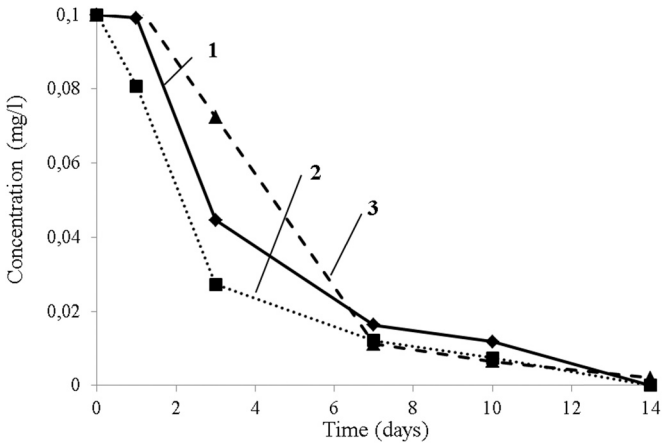


Fig. 5. Concentration for mixture M3 components as a function of biodegradation time under the action of bacterial strains of CH628 (1), KT112–7 (2) and P25 (3).

potential, leaving approximately 2% of M3 mixture unused for biodegradation in 14 days. Because of the co-elution of many components of the degraded mixture, it is not possible to provide more comprehensive information on the biodegradation rates for specific PCBs-OH and PCB congeners from mixture M3 under the action of bacterial strains.

The HPLC analysis revealed that polychloro- and hydroxy(polychloro)benzoic acids are accumulated in the media as the main products of M3 mixture biodegradation. The HPLC analysis at a wavelength of 232 nm within the UV range, which is characteristic of polychlorobenzoic acids, six peaks of compounds were recorded; and the analysis at a wavelength of 205 nm, which is characteristic of hydroxy (polychloro)benzoic acids, eight peaks of compounds. Therefore, it was established that polychloro- and hydroxy(polychloro)benzoic acids are formed as metabolites of mixture M3. Fig. 6 displays the formation of polychloro- and hydroxy(polychloro)benzoic acids depending on various strain destructor. It was found that in all the cases the formation of polychlorobenzoic acids prevailed.

The obtained results allow for an assumption to be made that in the mixture M3 the strains KT112–7, P25 and CH628 destruct unreacted PCBs more actively than PCBs-OH. That is the reason for a considerable amount of polychlorobenzoic acids formed. However, because the fraction of PCBs-OH in mixture M3 is greater than the fraction of unreacted PCBs, then the contribution made by hydroxy(polychloro) benzoic acids as metabolites should also be considerable. Nevertheless, the analysis revealed that the total content of hydroxy(polychloro) benzoic acids was lower than the total content of polychlorobenzoic acids. This fact allows a suggestion to put forward that the strains KT112–7, P25 and CH628 are capable destructors of hydroxylated chlorobenzoic acids.

In the process of studying the biodegradation, it was found that the strains KT112–7, CH628 and P25, apart from the sufficiently well expected destructor's function in relation to mixture M3, can be used as a source of carbon in the process of cellular growth (Fig. 7). It is likely that the comparatively low growth rate of the strain KT112–7 is due to the fact that the enzymes degrading chlorobenzoic acids down to the compounds involved in the anabolic processes of a cell show little activity at the initial stages.

It was also established that the process of biodegradation of mixture M3 was accompanied with chlorine atoms being released into the medium: for CH628 strain, up to 2.2% of the maximally possible amount; for KT112–7, up to 6.8% of maximally possible amount; and for P25 strain, up to 22.1% of the maximally possible amount (Table S2).

Taking into consideration the results obtained in the study, and also accounting for the results found earlier (Tehrani et al., 2012; Mizukami-Murata et al., 2016; Plotnikova et al., 2012), a conclusion can

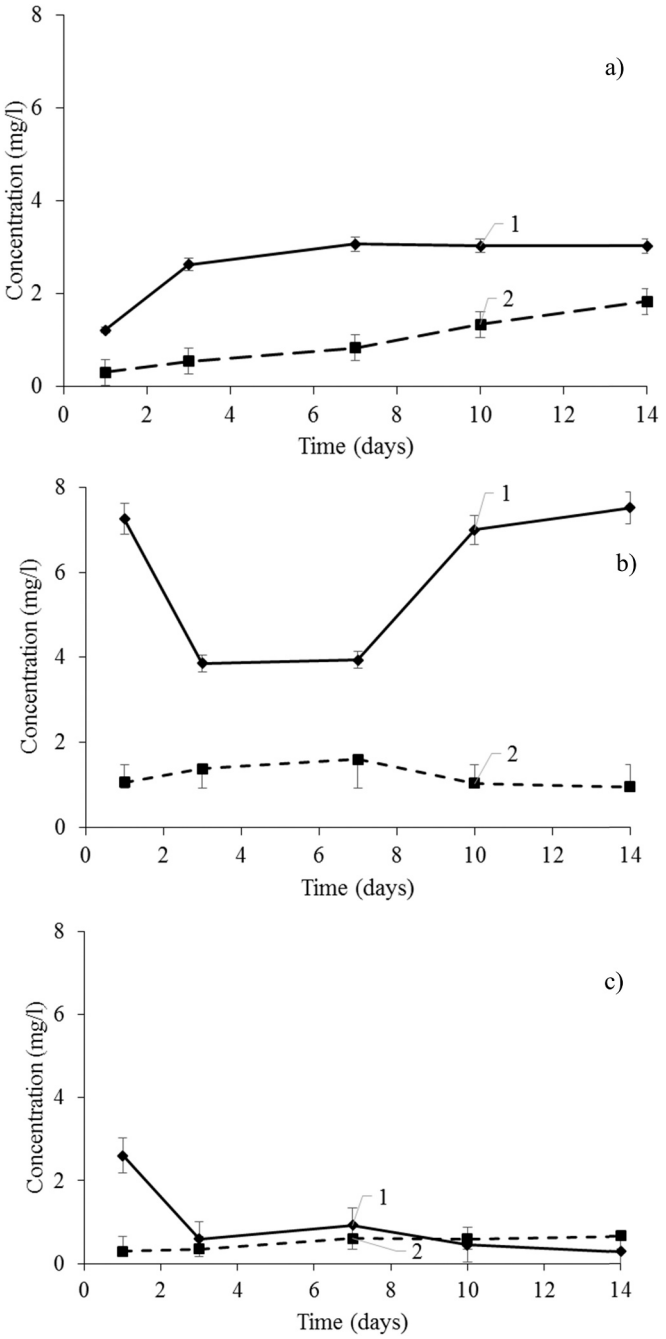


Fig. 6. Concentrations for polychloro- (1) and hydroxypolychlorobenzoic (2) acids as a function of time of biodegradation of mixture M3 under the action of bacterial strains of CH628 (*a*), KT112–7 (*b*) и P25 (*c*).

be made that biphenyl dioxygenase of the strains oxidizes the least substituted ring as in the mixture PCBs-OH as in the unreacted PCBs present in mixture M3.

When studying the metabolites of mixture M3, there was no accumulation of the compounds of structure C found in the medium. Apparently, the action of enzymes of the entire pathway is coordinated, which allows the strains CH628, KT112–7 and P25 to destruct the PCBs and PCBs-OH mixture effectively.

4. Conclusions

The results obtained in this study demonstrate the behavior of trichlorobiphenyls, their hydroxy derivatives and mixtures of PCBs and

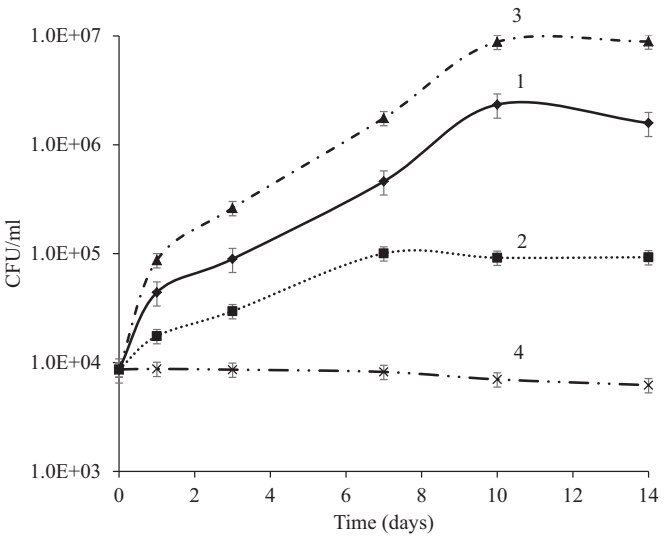


Fig. 7. C H628 (1), KT112–7 (2) and P25 (3) strains growing in K1 mineral medium in biodegradation conditions with mixture M3 as a sole carbon source. Bacterial strains under the same conditions without adding of mixture M3 (4).

PCBs-OH under the action of aerobic strain of *Rhodococcus*; the results are of significance, because the experimental works are, in fact, simulating the transformations of PCBs and PCBs-OH in the environment. The features revealed in the biodegradation of PCBs and PCBs-OH can be adapted to become applicable for the PCBs mixture Aroclor 1242 (USA), Delor 103 (Czechoslovakia) and for their hydroxylated derivatives.

The performed research works have allowed a number of important and significant points to be made:

1. the experimental data based on the study of hydroxylation of individual trichlorobiphenyls and technical mixture PCB Trikhlorbifenil, allow a statement to be made that the period needed for converting PCBs into PCBs-OH in the environment is specific to every congener and is determined with the number of chlorine atoms in the biphenyl structure and their position in two aromatic rings;
2. in the presence of aerobic strains CH628, KT112-7 and P25, the time required for biodegradation of individual PCBs-OH and their mixture also depends on the structural peculiarities of the initial compounds;
3. the studied microorganisms are considered to be effective biodestructors of individual PCBs, PCBs-OH and their versions as mixtures with a variety of components;
4. there was no fundamental features revealed for the bacterial degradation of PCBs and PCBs-OH under the action of the strains CH628, KT112-7 and P25. Polychloro- and hydroxy(polychloro)benzoic acids were found to be the identified final metabolites of PCBs and PCBs-OH biodegradation, which allows a correlation to be made between the obtained results and the conventional opinion on further degrading of the acids revealed down to the products of the base exchange reaction in a cell: water, carbon dioxide and chlorine compounds;
5. the CH628, KT112-7 and P25 bacterial strains are capable of becoming exogenic material to be used for remediation of soil and water contaminated with persistent organic pollutants of chloroaromatic origin;
6. the revealed specific features of chemical functionalization of PCBs are characteristic of low-chlorinated congeners only, and the established regularities of biodegradation of PCBs and PCBs-OH can be relevant for *Rhodococcus*-strains only. Any other chemical and bacterial matters call for further separate studies.

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The gaseous chromatography analysis was carried out using the equipment at the Spectroscopy and Analysis of Organic Compounds Center of Joint Use.

CRediT authorship contribution statement

Tatyana I. Gorbunova: Conceptualization, Investigation, Formal analysis, Validation, Writing - original draft. **Darya O. Egorova:** Conceptualization, Investigation, Formal analysis, Validation, Writing - original draft, Funding acquisition. **Marina G. Pervova:** Conceptualization, Investigation, Formal analysis, Validation, Visualization, Writing - original draft. **Tatyana D. Kyrianova:** Investigation, Formal analysis. **Vitalyi A. Demakov:** Writing - review and editing, Project administration. **Victor I. Saloutin:** Writing - review and editing, Project administration. **Oleg N. Chupakhin:** Writing - review and editing, Supervision.

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.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2020.124471.

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