Chapter 1

Bioremediation of Chlorobenzoic Acids

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Additional information is available at the end of the chapter

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1. Introduction

Chlorobenzoic acids (CBAs) can be released into the environment from many different sources. One possible source of CBAs is usage as herbicides or insecticides in agriculture. As a herbicide was used 2,3,6-CBA [1]. CBAs may also be formed as intermediates during the degradation of same herbicides. Namely 2,6-CBA is formed as an intermediate during microbial degradation of dichlobenil [2], 2,5-CBA in the chlorambene degradation [3] or 4-CBA is a final degradation product of the insecticide DDT [4].

Another large group of substances from whose metabolism in living organisms including mammals arise CBAs, are drugs such as indomethacin, bupropion or benzafibrate. Indomethacin is an anti-inflammatory drug used to reduce fever, pain, stiffness and swelling by inhibition of the production of prostaglandins, compounds that cause these problems. From the decomposition of indomethacin arises 5-methoxy-2-methylindoleacetic acid and the same quantity of 4-CBA [5]. In the case of antidepressant bupropion just small amount (0.3%) of 3-CBA is formed next to threohydrobupropion, erythrohydrobupropion and hydroxybupropion [6, 7]. Decomposition of bezafibrate, an anti-obesity drug, leads to the formation of equal quantity of 4-CBA and 4-(2-aminoethyl)-α,α dimethyl-benzeneacetic acid. [8].

Well known is the formation of CBAs during degradation of polychlorinated biphenyls (PCBs). PCBs are degraded by bacteria by the so called upper degradation pathway when CBAs are formed as final degradation products (Figure 1.) [10-12]. CBAs are also formed during degradation of PCBs by white rot fungi [13] [14]. The result of these metabolic pathways is a mixture of CBAs with different position and number of chlorine atoms on benzene ring in dependence of chlorination of the degraded PCB congeners [9].

The accumulation of this way formed CBAs in waste water or in soil can lead to the deceleration or inhibition of degradation of substances of which the CBAs are degradation products [15,

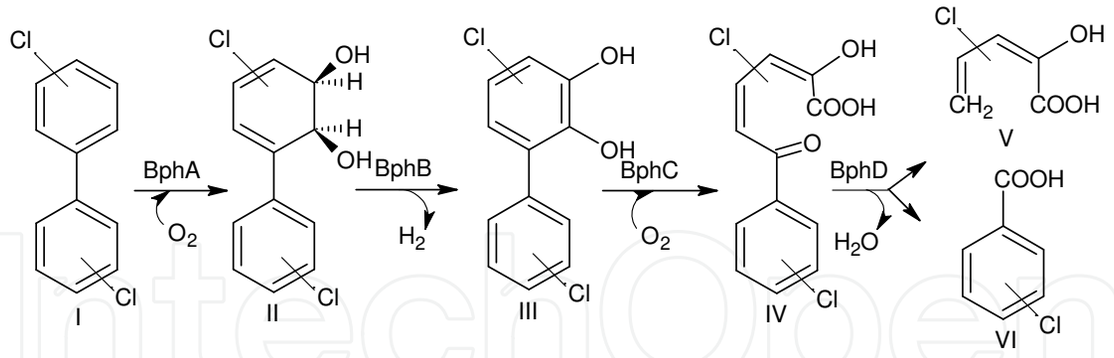

Figure 1. Degradation pathway of polychlorinated biphenyls by aerobic organisms [9]. BphA – biphenyl-2,3-dioxygenase; BphB – biphenyl-dihydrodiol-dehydrogenase; BphC – 2,3-dihydroxy-biphenyl-1,2-dioxygenase; BphD – 2-hydroxychlor-6-oxo-6-phenylhexa-2,4-dienoathydrolase; I – polychlorinated biphenyl; II – dihydrodiol; III – 2,3-dihydroxychlor biphenyl; IV – 2-hydroxychlor-6-oxo-6-phenylhexa-2,4-dienoic acid; V – chlorobenzoic acid; VI – 2-hydroxychlor-2,4-dienoate

16]. Therefore, it is important to understand bioremediation mechanisms of CBAs and to know the impact of other organisms, xenobiotics composition and the influence of other CBA isomers present in contaminated area that affect this process.

2. Bacterial degradation of chlorobenzoic acids

Bacterial degradation of CBAs can be done under aerobic or anaerobic conditions. Under anaerobic conditions is dechlorination the first step of degradation followed by degradation of the aromatic ring [17, 18].

The strategy of CBAs degradation under aerobic conditions depends on the position of chlorine atom or atoms on the aromatic ring. Crucial step is the dechlorination. Dechlorination step can be before or after degradation of aromatic ring. This depends on the structure of CBA as well as on the enzymatic equipment of the bacteria.

2.1. Anaerobic degradation of chlorobenzoic acids

Initial step of microbial anaerobic degradation of CBAs is done by process named reductive dehalogenation. During the dehalogenation are from the more chlorinated CBA by dehydrogenation process formed less chlorinated CBAs or benzoic acid and chlorine is released as hydrochloric acid [19]. Time scale of this process depends on the number and position of the chlorine atoms in the molecule of CBA and on composition of anaerobic consortium or on strain with dehalogenation activity. Dehalogenation of CBAs can be performed under methanogenic [20], denitrification [21] or sulfate reducing conditions [22]. Also phototrophic bacteria capable of reductive dehalogenation of CBAs are known, e. g. *Rhodopseudomonas palustris* DCP3 [23], strain able to degrade under anaerobic conditions 2-CBA; 3-CBA; 4-CBA and 3,4-CBA. This strain is also capable of anaerobic degration of benzoic acid.

The next step after reductive dehalogenation is the degradation of benzoic acid. Benzoic acid can be anaerobically degraded by two different pathways [24]. The first one is initiated by reduction of benzoic acid aromatic ring. Subsequent ring cleavage and degradation of intermediates occurs by reactions similar to β-oxidation of aliphatic carboxylic acids [25].

In the second pathway is the molecule of benzoic acid activated by binding to the CoA. CoA remains bound to the molecule during all degradation steps. In this pathway is benzoate-CoA degraded to the acetyl-CoA [26]. Acetyl-CoA is then degraded in tricarboxylic acid cycle to the CO_2 and water.

From previous it can be concluded that in anaerobic degradation is the molecule of CBA degraded to methane, CO_2 and water with benzoic acid as intermediate (Figure 2.) [17].

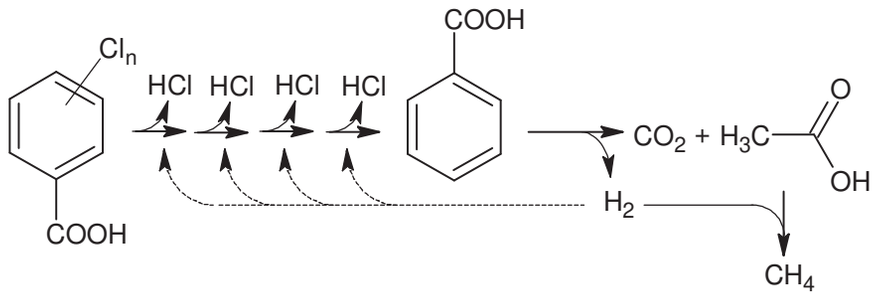

Figure 2. Anaerobic degradation of chlorobenzoic acid adapted from [17].

Anaerobic degradation of CBAs can be done by pure cultures of microorganisms as well as by consortia of anaerobic microorganisms. The first pure strain that has been observed to be able to anaerobically degrade CBA was strain *Desulfomonile tiedjei* [27]. This strain is capable of 3-CBA dehalogenation, by this process reaching enough ATP for its growth. So it does not need additional source of energy. Another strain able to anaerobic degradation of CBA is strain *Desulfomicrobium escambiense* [22] and the aforementioned strain *Rhodopseudomonas palustris* DCP3 [23].

2.2. Aerobic degradation of chlorobenzoic acids

Aerobic microorganisms evolved many different degradation strategies for CBAs degradation. Microbial aerobic degradation of CBAs depends on bacterial strain as well as on the structure and chlorination of CBA.

CBAs can be degraded via chlorocatechole (*clc* degradation genes) [28, 29] or by hydrolytic dehalogenation with hydroxybenzoic acid as an intermediate like in 4-CBA degradation (*fcb* degradation genes) [30-32] or by 4,5-dioxygenation like in 3-CBA and 3,4-CBA degradation where is 5-chloroprotocatechuic acid formed (*cba* degradation genes) [33, 34] or by 1,2-dioxygenase reaction [9, 35] or by degradation involving the formation of gentisate as an intermediate of CBA degradation [36, 37].

Strains able to aerobically degrade CBAs belong to the gram negative strains as *Rhodococcus* or *Bacillus* as well as gram positive strains like *Pseudomonas, Burkholderia* or *Achromobacter* [9].

2.2.1. Degradation of 2-chlorobenzoic acid

In case of 2-CBA degradation there are known three different ways of degradation. All of them are about dioxygenation reaction catalyzed by 2-halobenzoate-1,2-dioxygenase (EC 1.14.12.13) (Figure 3.).

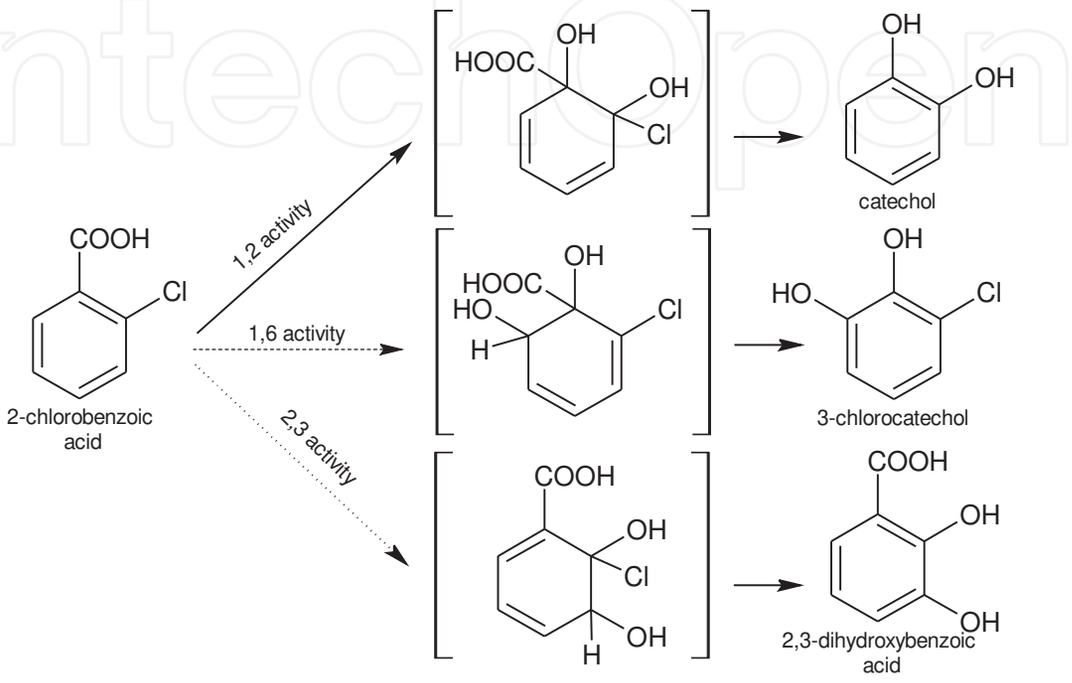

Figure 3. Aerobic degradation of 2-chlorobenzoic acid by the enzyme 2-chlorobenzoate-1,2-dioxygenase [9, 38].

Main activity of 2-halobenzoate-1,2-dioxygenase is 1,2-dioxygenase reaction. In this reaction is 2-CBA degraded to catechol. During this reaction are released carbon dioxide and chlorine. Enzyme 2-halobenzoate-1,2-dioxygenase has also 1,6-dioxygenase activity. In 1,6-dioxygenase reaction is formed 3-chlorocatechol. Last activity of 2-halobenzoate-1,2-dioxygenase is 2,3-dioxygenase reaction leading to the formation of 2,3-dihydroxybenzoic acid. Latter two reactions proceed only in a small degree [9].

Degradation of 2-CBA by 2-halobenzoate-1,2-dioxygenase was reported in two groups of microorganisms. First group degraded only 2-CBA and second next to 2-CBA also 2,3-CBA and 2,5-CBA. Both groups use for degradation 1,2-dioxygenase reaction. In case of 2-CBA this reaction leads to the formation of catechol (Figure 3.). 2,4-CBA and 2,5-CBA are degraded into 4-chlorocatechol (Figure 4.) [35].

For the 4-chlorocatechol degradation formed in 1,2-dioxygenase reaction with 2,4-CBA or 2,5-CBA it is necessary to have chlorocatechol degradation pathway. It is therefore assumed that the difference between both groups of microorganisms is given by presence of the chlorocatechol degradation pathway [9] despite the fact that two different 2-halobenzoate-1,2-dioxygenases are known.

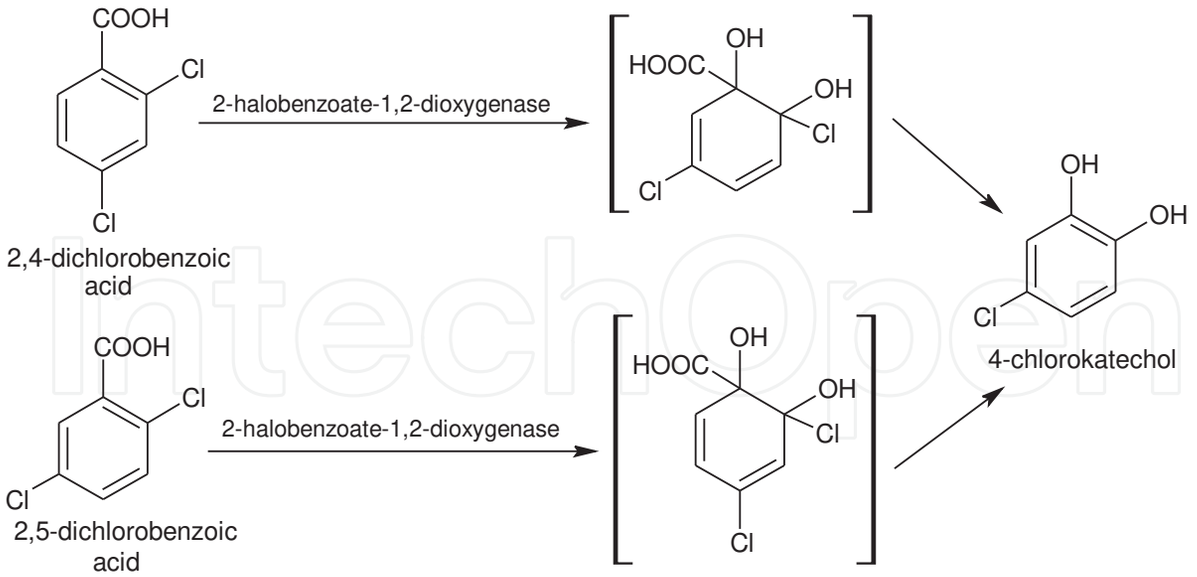

Figure 4. Degradation of 2,4-dichlorobenzoic and 2,5-dichlorobenzoic acid catalyzed by 2-halobenzoate-1,2-dioxygenase.

First 2-halobenzoate-1,2-dioxygenase is a two componential enzymatic system [39]. This 2-halobenzoate-1,2-dioxygenase has high homology with the toluate and benzoate-1,2-dioxygenase. Two component 2-halobenzoate-1,2-dioxygenase has high affinity to 2-CBA but low to 4-CBA; 2,4-CBA and 2,5-CBA[9, 40].

Second 2-halobenzoate-1,2-dioxygenase is three componential [35] and this enzyme catalyzes degradation of 2-CBA; 2,4-CBA and 2,5-CBA.

Next to 1,2-dioxygenase activity the enzyme 2-halobenzoate-1,2-dioxygenase has also 1,6-dioxygenase activity [9, 41]. Strain *Pseudomonas* sp. B-300 was in presence of glucose able to degrade 2-CBA to the 3-chlorocatechol [41].Whereas *Pseudomonas* sp. B-300 was cultivated with 2-CBA as the only carbon and energy source just catechol was identified. From that it is obvious that 1,6-dioxygenase reaction occurs only in much smaller degree than 1,2-dioxygenase reaction or in the presence of a rich source of energy.

Dioxygenase reaction in the 1,6- position is followed by 3-chlorocatechol ring cleavage forming chloromuconic acid which is dehalogenated.

Another metabolite identified in medium after cultivation of strain *Burkholderia cepacia* 2CBS with 2-CBA was 2,3-dihydroxybenzoic acid [42]. Strain *Burkholderia cepacia* 2CBS degraded most of 2-CBA by 1,2-dioxygenase reaction, 2,3-dihydroxybenzoic acid was in medium accumulated just in small quantities. Formation of 2,3-dihydroxybenzoic acid indicates that two-component 2-halobenzoate-1,2-dioxygenase of strain *Burkholderia cepacia* 2CBS has next to 1,2-dioxygenase activity also 2,3-dioxygenase activity (Figure 3.). 2,3-dihydroxybenzoic acid is the dead-end product [9].

2.2.2. Degradation of 3-chlorobenzoic acid

Degradation of 3-CBA can be done by several different degradation pathways. Benzoate-1,2-dioxygenase catalyzes the conversion of 3-CBA into 3-chlorocatechol or 4-chlorocatechol. 3-CBA can be also transformed to the protocatechuate (3,4-dihydroxybenzoic acid) or 5-chloroprotocatechuate (5-chloro-3,4-dihydroxybenzoic acid) by the enzyme 3-chlorobenzoate-4,5-dioxygenase. Another possibility is the degradation of 3-CBA via 3-hydroxybenzoic acid to the gentisic acid (2,5-dihydroxybenzoic acid).

First mentioned 3-CBA degradation is degradation in the benzoate degradation pathway. Breakdown of 3-CBA is catalyzed by the enzyme benzoate-1,2-dioxygenase (EC 1.14.12.10). Benzoate-1,2-dioxygenase is a wide spread enzyme which has been identified in many microorganisms. Substrate specificity of this enzyme is relatively narrow. Benzoate-1,2-dioxygenasa catalyzes only conversion of benzoate, 3-CBA and 3-methylbenzoate [9, 43, 44].

The mechanism of reaction catalyzed by the benzoate-1,2-dioxygenase is based on the double hydroxylation in 1,2- or 1,6-position of benzene ring. The final product of this reaction is catechol in case of benzoic acid or 4-chlorocatechol or 3-chlorocatechol in case of 3-CBA degradation (Figure 5.).

Benzoate-1,2-dioxygenase of the strain *Alcaligenes eutrophus* JMP134 [43] form from 3-CBA 3-chlorocatechol and 4-chlorocatechol in a 1:2 ratio, as well as the benzoate-1,2-dioxygenase of strain *Pseudomonas* sp. B13 [44] do.

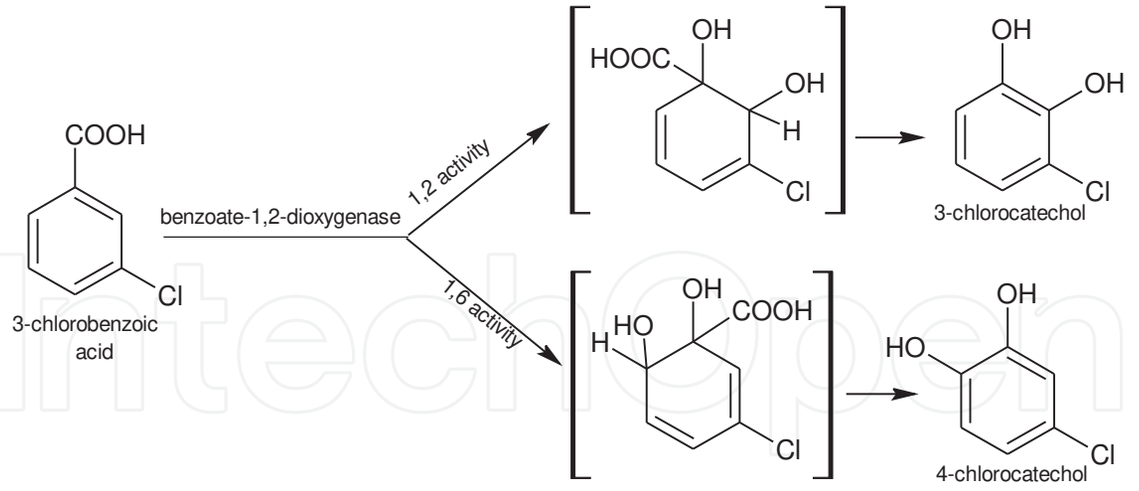

Figure 5. Degradation pathway for 3-chlorobenzoic acid by benzoate-1,2-dioxygenase [9] with indicated 1,2-dioxygenase and 1,6-dioxygenase activity.

Next to benzoate-1,2-dioxygenase 3-CBA can be degraded also by the 3-chlorobenzoate-3,4-dioxygenase [45-47]. Main activity of this enzyme is 4,5-dioxygenase reaction with formation of 5-chloroprotocatechuic acid. Besides the 4,5-dioxygenase reaction is a small amount of 3-CBA transformed by 3,4-dioxygenase reaction to the protocatechuic acid (Figure 6.).

The enzyme 3-chlorobenzoate-3,4-dioxygenase can catalyze also degradation of 3,4-CBA. 3,4-CBA is degraded to the 5-chloroprotocatechuic acid [34, 48] which means that 3,4-CBA is degraded by the 4,5-dioxygenase reaction.

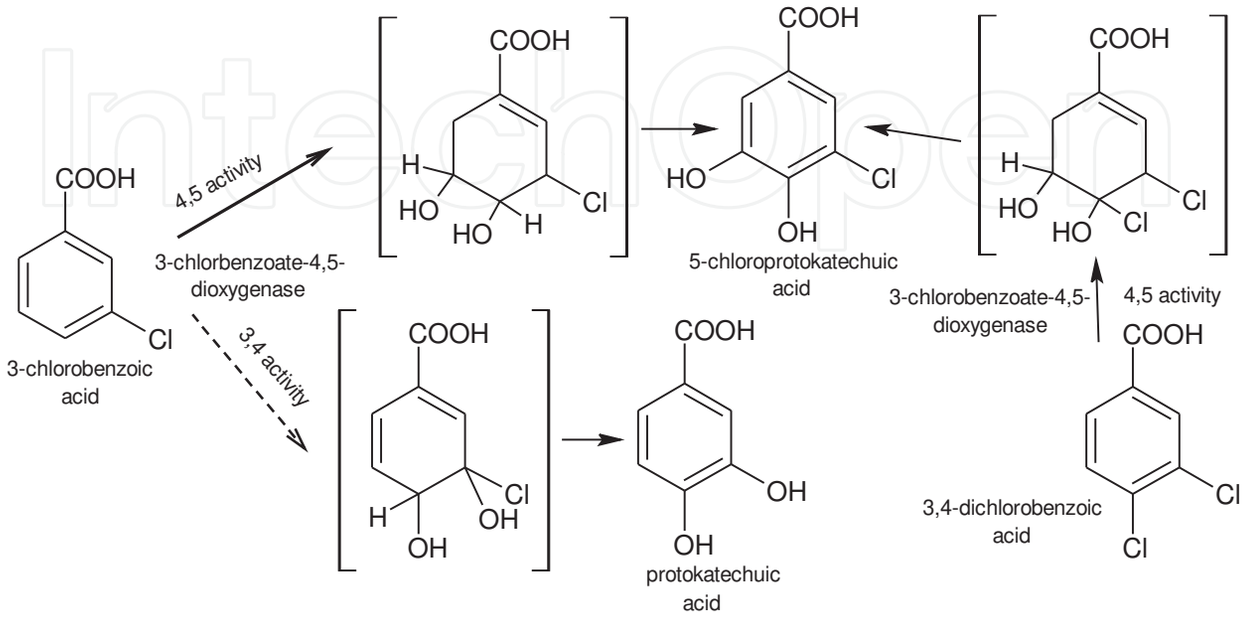

Figure 6. Degradation pathway of 3-chlorobenzoic and 3,4-dichlorobenzoic acid by 3-chlorobenzoate-3,4-dioxygenase.

3-CBA can be also degraded by monooxygenase reaction. This reaction leads to the gentisic acid with 3-hydroxybenzoic acid as an intermediate (Figure 7.) [37]. The enzyme for the conversion of 3-CBA to the 3-hydroxybenzoic acid is not yet known, the second reaction is catalyzed by 3-hydroxybenzoate-6-hydroxylase (EC 1.14.13.24) [49].

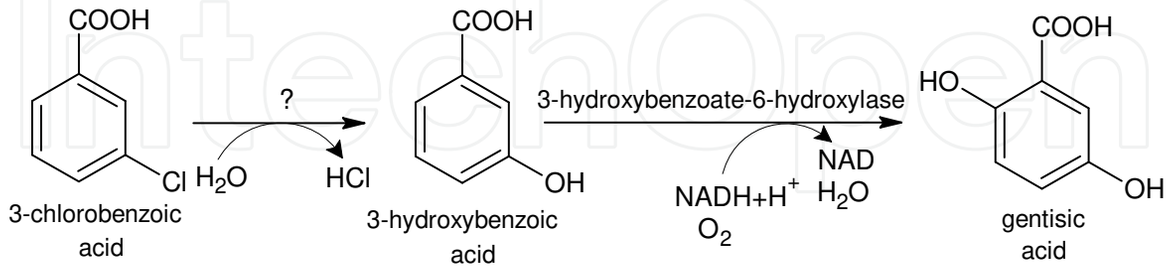

Figure 7. Degradation pathway of 3-chlorobenzoic acid with gentisic acid as an intermediate.

Monooxygenase reaction was reported in strains *Pseudomonas* sp. [50] and *Alcaligenes* sp. L6 [37]. Strain *Alcaligenes* sp. L6 has next to this pathway also pathway for 3-CBA degradation with protocatechuic acid as the final product. Strain L6 was isolated under low oxygen concentration. From this it can be assumed that 3-CBA degradation by monooxygenase

reaction is held only under low oxygen concentration. When such conditions do not occur, the preferred microorganisms are those which can 3-CBA degrade by dioxygenase reaction. It may be also the reason why are described only a few strains degrading 3-CBA via gentisic acid.

2.2.3. Degradation of 4-chlorobenzoic acid

Microorganisms able of the degradation of 4-CBA mostly belong to the strains *Alcaligenes, Arthrobacter* and *Pseudomonas* [51]. Until now, two pathways for 4-CBA degradation were described. In first, the more common, is 4-CBA dehalogenation followed by ring cleavage. In the second is 4-CBA converted to 4-chlorocatechol which is further subjected to the ring cleavage and only then is dehalogenated [52].

In first mentioned 4-CBA degradation pathway is 4-hydroxybenzoic acid formed (Figure 8.). This pathway begins with the conversion of 4-CBA to 4-chlorobenzoate-CoA catalyzed by 4-chlorobenzoate:CoA ligase with the consumption of 1 molecule of ATP [53]. This reaction is followed by replacement of chlorine atom with hydroxyl group derived from water catalyzed by 4-chlorobenzoate:CoA dehalogenase [54]. The last step of 4-CBA dehalogenation is the hydrolysis of 4-hydroxybenzoate-CoA thioester by the enzyme 4-hydroxybenzoate:CoA thioesterase with formation of 4-hydroxybenzoate [32].

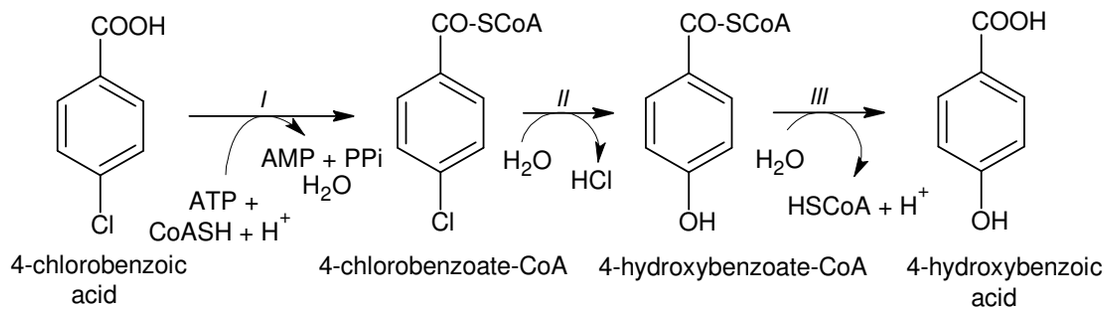

Figure 8. Degradation pathway of 4-chlorobenzoic acid with formation of 4-hydroxybenzoic acid. *I* – 4-chlorobenzoate:CoA ligase; *II* - 4-chlorobenzoate:CoA dehalogenase; *III* - 4-hydroxybenzoate:CoA thioesterase

All of the enzymes required for this conversion are organized in one operon. This operon is regulated by the presence of 4-CBA. Genes coding this operon can be located in the chromosomal DNA as well as on a plasmid [32].

The order of genes in the operon is for each bacterial strain different. *Pseudomonas* strain has dehalogenase-ligase-thioesterase and strain *Arthrobacter* has ligase-dehalogenase-thioesterase [32]. This indicates that the genes coding dehalogenation of 4-CBA have been rearranged. The low agreement of protein sequences indicates that this pathway is not the result of a recent adaptation. Therefore it is not the result of the recent release of PCBs and thus also 4-CBA, but it can be assumed that the 4-CBA dehalogenation pathway had enough time to arise from random mutations and selections. Indirectly it can be assumed that there is a natural source of 4-CBA [55].

Temporary formation of CoA thioester, one step after it was created, is special due to the energy consuming production of thioester bond. At the same time thioester formation is not required for aerobic degradation of other CBAs.

In case of anaerobic degradation of 3-CBA by strain *Rhodopseudomonas palustris* RCB100 is 3-chlorobenzoate-CoA formed. Its formation is followed by dehalogenation with release of benzoate-CoA. Benzoate-CoA is further degraded to the acetyl-CoA [56]. Same pathway is used for benzoic acid degradation. This pathway begins with formation of benzoate-CoA and CoA remains bound through the whole degradation pathway [24]. For the aerobic degradation of 4-CBA is thioester required only for the efficient dehalogenation. Thus, consumption of one molecule of ATP is acceptable due to the fact that it allows degradation of the molecule and enables its use as a source of carbon and energy.

The second possibility of 4-CBA degradation is degradation with 4-chlorocatechol as an intermediate (Figure 9.). Formation of 4-chlorocatechol is followed by ring cleavage and dehalogenation. This way of 4-CBA degradation is much less investigated than 4-CBA degradation leading to the formation of 4-hydroxybenzoate.

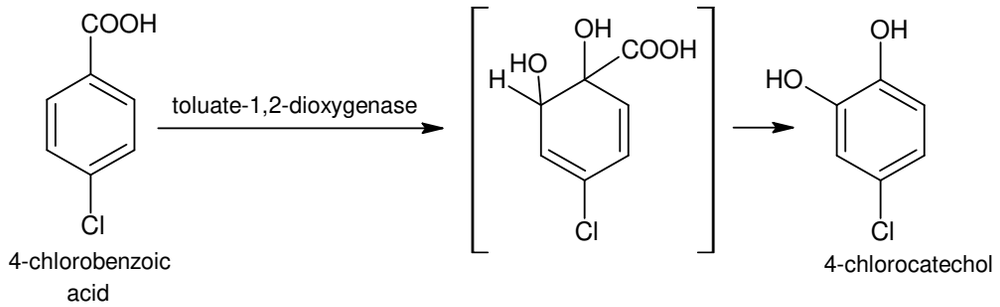

Figure 9. Degradation pathway of 4-chlorobenzoic acid leading to the 4-chlorocatechol

Enzyme of first step of 4-CBA degradation to the 4-chlorocatechol can be either the toluate-1,2-dioxygenase (EC 1.14.12.10) [31] like in case of strain *Pseudomonas aeruginosa* mt-2 or by not yet closer identified chlorobenzoate-1,2-dioxygenase described for example in the strain *Pseudomonas aeruginosa* 3mT [36].

Strain *Pseudomonas aeruginosa* 3mT was also able to degrade 3-CBA with formation of 3-chlorocatechol but formation of 4-chlorocatechol was not recorded. This activity suggest that *Pseudomonas aeruginosa* 3mT dioxygenase has only 1,2-dioxygenase activity. Not like benzoate-1,2-dioxygenase and 2-halobenzoate-1,2-dioxygenase 1,2- and 1,6-dioxygenase activity. From this can be assumed that dioxygenase from strain *Pseudomonas aeruginosa* 3mT is not identical with either of these enzymes.

2.2.4. Degradation of more chlorinated chlorobenzoic acids

Aerobic degradation of CBA with two chlorine atoms in molecule has been reported for many strains [17]. Degradation of CBA with three chlorine atoms is relatively rare, in the literature

is described only a few strains and in most cases CBAs with three chlorine atoms in molecule are degraded cometabolically.

Dichlorinated CBA can be degraded by a dioxygenase reaction. For example 2,4-CBA and 2,5-CBA which are degraded by three componential 2-halobenzoate-1,2-dioxygenase with release of 4-chlorocatechol, as described in section 2.2.1. Also degradation of 3,4-CBA catalyzed by 3-chlorobenzoate-3,4-dioxygenase with formation of 5-chloroprotocatechuate has been previously described in section 2.2.2.

In case of 2,4-CBA is known one more degradation pathway similar to the 4-CBA degradation described in section 2.2.3. In this pathway is 2,4-CBA degraded via 4-hydroxybenzoate to the protocatechuic acid (Figure 10.). This pathway has been described in strains *Corynebacterium sepedonicum* KZ-4 [57] and *Alcaligenes denitrificans* NTB-1 [58].

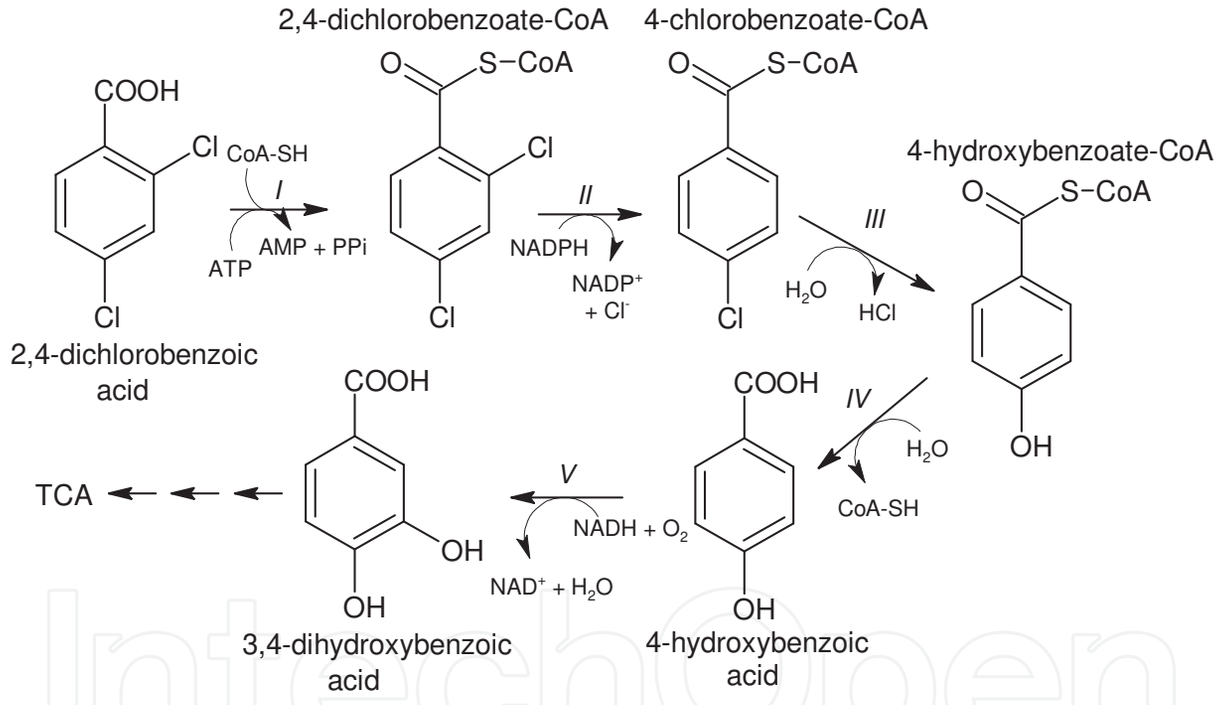

Figure 10. Degradation pathway of 2,4-dichlorobenzoic acid by strain *Corynebacterium sepedonicum* KZ-4 [57]. *I* - 2,4-dichlorobenzoate:CoA ligase; *II* – 2,4-dichlorobenzoate:CoA reductase; *III* – 4-chlorobenzoate:CoA dehalogenase; *IV* – 4-chlorobenzoate:CoA thioesterase; *V* – 4-hydroxybenzoate 3-monooxygenase; TCA – tricarboxylic acid cycle

Strain *Pseudomonas* sp. WR912 [59] can degrade 3,5-CBA by 1,2-dioxygenase reaction with release of 3,5-dichlorocatechol, which was by the same strain degraded to the succinate. Another way of 3,5-CBA degradation is the cometabolic degradation by consortia 2m.c [60]. 3,5-CBA was cometabolically converted to the 3,5-dichlorocatechol as a final intermediate by enzyme 2-halobenzoate-1,2-dioxygenase with presence of 2-CBA and 2,5-CBA. Presence of 3,5-CBA leads to the loss of 2-CBA degradation ability of the 2m.c consortium.

Same 2m.c consortium can also cometabolically degrade 2,3-CBA via 3-chlorocatechol to the 2-chloromuconic acid with presence of 2-CBA [60]. Strain *Pseudomonas aeruginosa* JB2 [61] degraded 2,3-CBA to the 4-chlorocatechol. Strain *Pseudomonas aeruginosa* JB2 can use also 2-CBA, 3-CBA, 2,5-CBA and 2,3,5-CBA as the sole carbon and energy source.

As it has been said before, CBAs with three chlorine atoms in molecule are converted in most cases cometabolically. Strain *Brevibacterium* sp. converted 2,3,6-CBA by a set of cometabolic steps to carbon dioxide and water [1]. This aerobic degradation proceeds via 2,3,6-trichloro-4-hydroxybenzoic acid or 2,3,6-trichloro-5-hydroxybenzoic acid to the 2,3,5-trichlorophenol or 2,4,5-trichlorophenol and finally to the 3,5-dichlorocatechol, which is than degraded by chlorocatechol degradation pathway (Figure 11.).

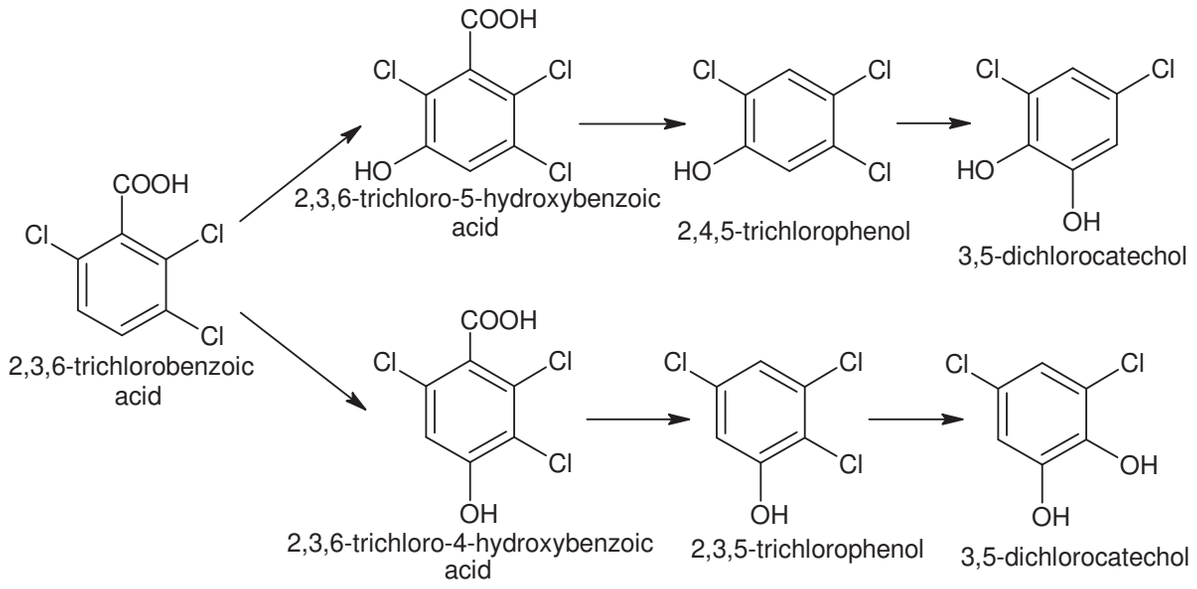

Figure 11. Cometabolic degradation of 2,3,6-trichlorobenzoic acid [1].

Strain *Pseudomonas putida* P111 degraded 2,3,5-CBA to the 3,5-dichlorocatechol (Figure 12.) but use different degradation pathway than strain *Brevibacterium* sp., 2,3,5-CBA was degraded by 1,2-dioxygenation followed by dehalogenation [62].

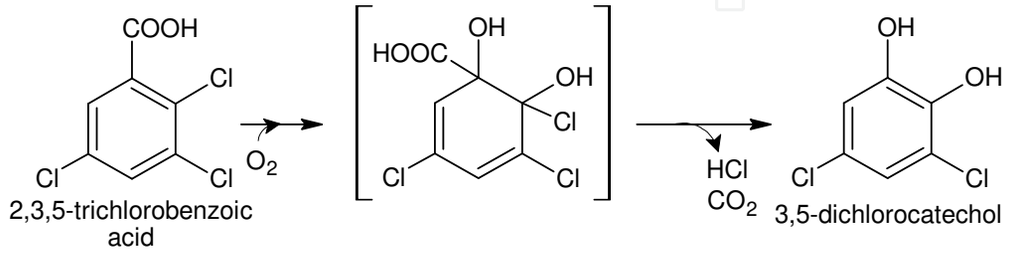

Figure 12. Degradation of 2,3,5-trichlorobenzoic acid by strain *Pseudomonas putida* P111 by 1,2-dioxygenase reaction [62].

2.2.5. Degradation of main chlorobenzoic acids degradation intermediates

In CBAs degradation are formed catechol, 3-chlorocatechol, 4-chlorocatechol, 4-hydroxybenzoic acid, gentisic acid, protocatechuic acid, chloroprotocatechuic acid and more chlorinated catechols.

Catechols can be degraded via *ortho-* or *meta-* cleavage pathway or possibly by modified *ortho-* cleavage pathway, which leads to the 3-oxoadipoic acid pathway. 3-oxoadipoic acid pathway is used for 4-hydroxybenzoic acid and protocatechuc acid degradation and leads to the tricarboxylic acid cycle.

In *ortho-* cleavage pathway is catechol or chlorocatechol degraded by catechol-1,2-dioxygenase or chlorocatechol-1,2-dioxygenase (Figure 13.). The first step of this pathway is dioxygenase reaction leading to the cleavage of the bond between the first and second position of the benzene ring. This reaction produces *cis,cis*-muconic acid or chloro-*cis,cis*-muconic acid with chlorine atom in a different position [63].

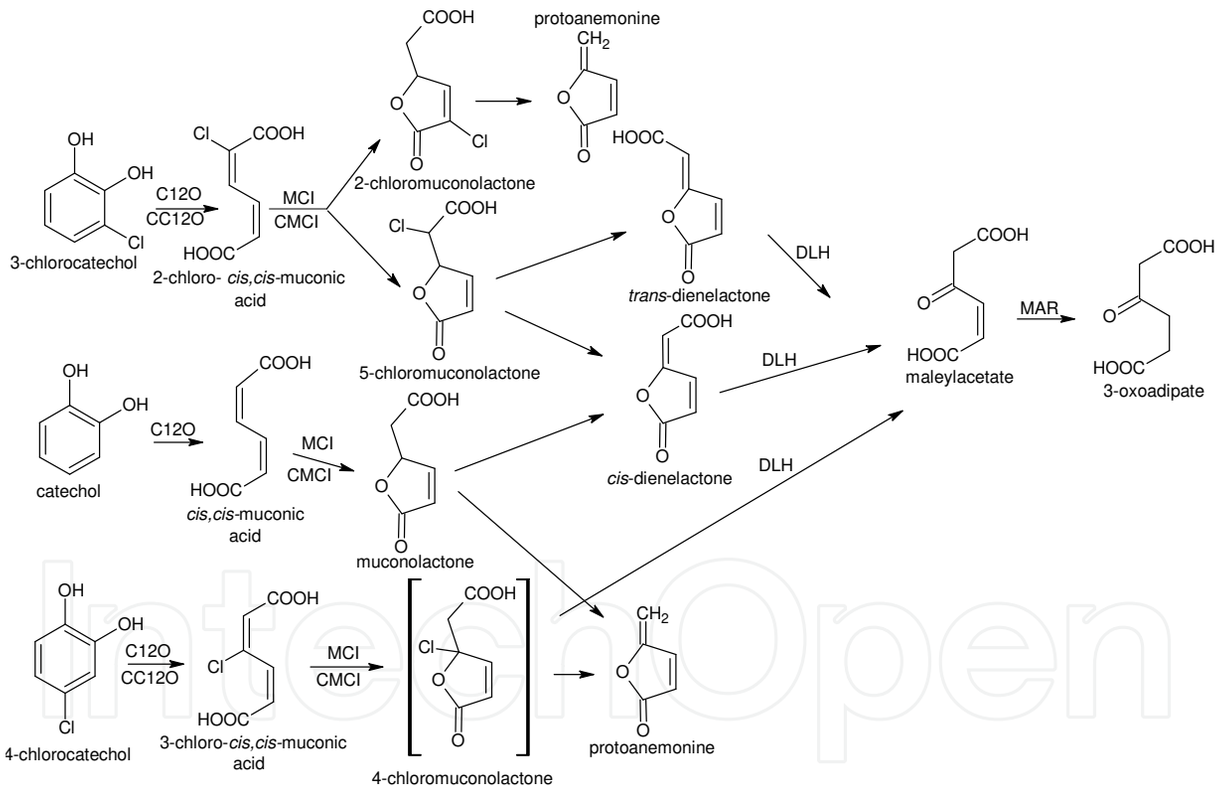

Figure 13. *Ortho-* and modified *ortho-* cleavage pathway of catechol or chlorotachols adapted from [9]. C12O – catechol-1,2-dioxygenase; CC12O – chlorocatechol-1,2-dioxygenase; MCI – muconate cycloisomerase; CMCI – chloromuconate cycloisomerase; DLH – dienelaktone hydrolase; MAR - maleylacetate reductase.

The second enzyme of this pathway is muconate cycloisomerase or chloromuconate cycloisomerase. This is a key enzyme in the degradation of chlorinated substances. The specificity of this isomerase determines whether degradation will be by *ortho-* cleavage pathway or by a modified *ortho-* cleavage pathway.

In *ortho*- cleavage pathway chloromuconate cycloisomerase catalyzes the conversion of chlorinated muconic acid to the *cis*- or *trans*-dienelactone in dependence of chlorine atom position in muconic acid. In this step is the molecule also dehalogenated [9].

Further enzyme of *ortho*- cleavage pathway is the dienelactone hydrolase. This enzyme is capable of conversion of *cis*- or *trans*-dienelactone to the maleylacetate. Maleylacetate is by maleylacetate reductase transformed into 3-oxoadipoic acid, which is further eliminated by 3-oxoadipoic acid degradation pathway (will be described later).

Majority of known strains, use for the catabolism of catechols or chlorocatechols a modified *ortho*- cleavage pathway [64]. In this pathway is chloro-*cis,cis*-muconate transformed by chloromuconate cycloisomerase to the protoanemonine (Figure 13.). Protoanemonine is a dead-end product of this pathway. Protoanemonine has also antimicrobial properties thus formation of protoanemonine from chlorinated catechols in modified *ortho*- cleavage pathway leads to the poor survival of degrading microorganisms in the soil.

Catechol or chlorocatechols can be also degraded by the enzyme katechol-2,3-dioxygenase or chlorocatechol-2,3-dioxygenase in *meta*- cleavage pathway which is initiated by this dioxygenation (Figure 14.). The result of dioxygenase reaction is the ring cleavage between second and third position of the benzene ring.

From catechol and 3-chlorocatechol is in this step 3-hydroxy-*cis,cis*-muconic acid formed. This acid is further degraded to the pyruvate and acetaldehyde [65]. The risk of this pathway is the possibility of formation of a reactive acylchloride from 3-chlorocatechol. Acylchloride irreversibly inactivate catechol-2,3-dioxygenase [66, 67].

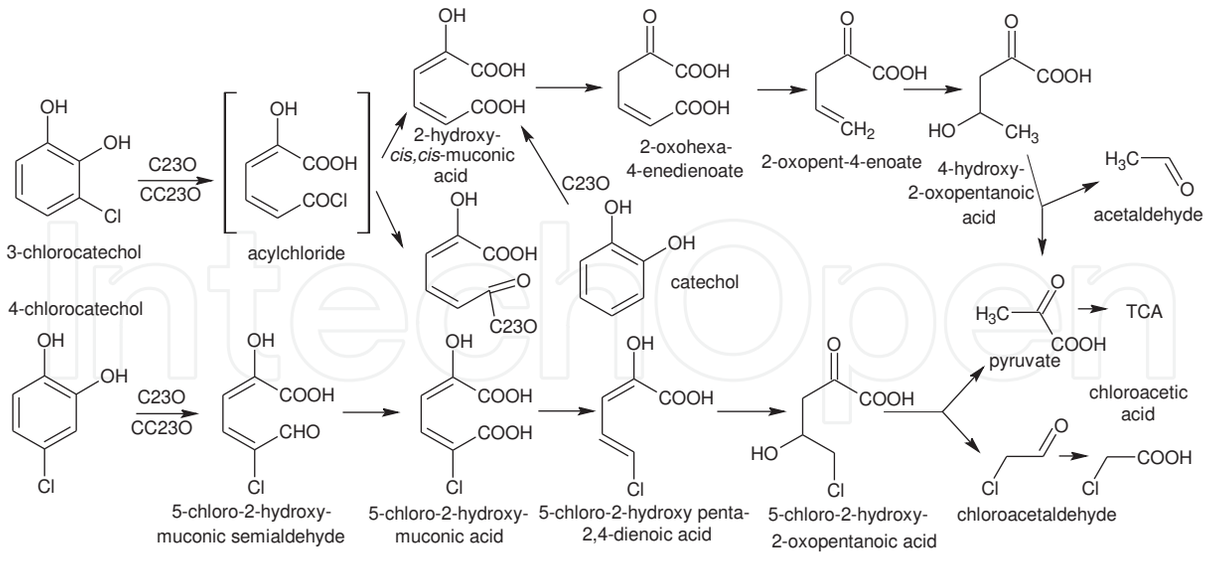

Figure 14. *Meta*- cleavage pathway of catechol or chlorotachols [9]. C23O - catechol-2,3-dioxygenase; CC23O - chlorocatechol-2,3-dioxygenase; TCA – tricarboxylic acid cycle.

The 4-chlorocatechol is by the 2,3-dioxygenase reaction converted to the 5-chloro-2-hydroxy-muconic semialdehyde [16]. This semialdehyde is in several steps transformed into the

pyruvate and chloroacetic acid. Pyruvate is further degraded in the tricarboxylic acid cycle. In case of chloroacetic acid is expected further degradation due to the fact that during the course of degradation of 4-CBA via 4-chlorocatechol by strain *Pseudomonas cepacia* P166 only a temporary accumulation of chloroacetic acid was registered [68].

In CBAs degradation next to catechol and chlorocatechols can be 4-hydroxybenzoic acid formed. 4-hydroxybenzoic acid is product of 4-CBA degradation (section 2.2.3). 4-hydroxybenzoic acid is further degraded in the 3-oxoadipoic acid degradation pathway (Figure 15.) [16]. The first step of this pathway is oxidation catalyzed by 4-hydroxybenzoate-3-monooxygenase with the formation of protocatechuic acid (3,4-dihydroxybenzoic acid). Benzene ring of protocatechuic acid is in next step cleaved between third and fourth position. This way formed 3-karboxy-*cis*,*cis*-muconic acid is than transformed into the 3-oxoadipoic acid [51, 69].

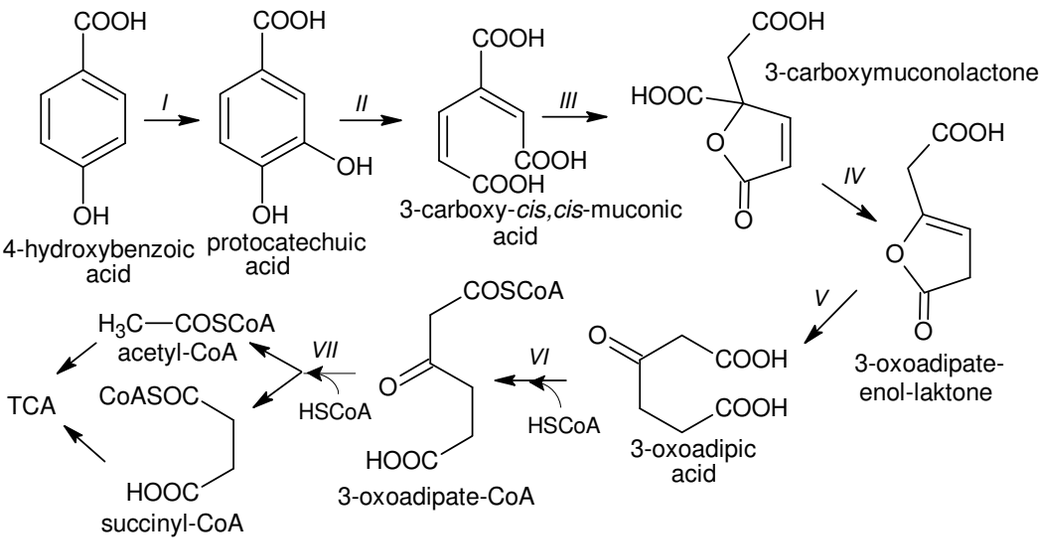

Figure 15. Degradation pathway of 3-oxoadipoic acid [9, 51, 69]. *I* – 4-hydroxybenzoate-3-monooxygenase; *II* – protokatechute-3,4-dioxygenase; *III* – 3-carboxymuconate-cykloisomerase; *IV* – 4-carboxymuconolaktone-dekarboxylase; *V* – 3-oxoadipate-enol-laktone hydrolase; *VI* - 3-oxoadipate:sukcinyl-CoA transferase; *VII* - 3-oxoadipate-CoA thiolase; TCA – tricarboxylic acid cycle.

3-oxoadipoic acid, which is also formed in the *ortho*- cleavage pathway from chlorinated catechols is then in two steps catalyzed by 3-oxoadipate:succinyl-CoA transferase and 3-oxoadipoate-CoA thiolase converted into the intermediates of the trikarboxylic acid cycle [9, 51, 69].

In 3-CBA degradation pathway initiated by monoxygenase reaction is gentisic acid formed (section 2.2.2). Gentisic acid (2,5-dihydroxybenzoic acid) is, as well as protocatechuic acid, degraded in the 3-oxoadipoic acid degradation pathway into the intermediates of the tricarboxylic acid cycle (Figure 16.).

The first step in gentisic acid degradation is the formation of maleylpyruvate by dioxygenase reaction catalyzed by gentisate-1,2-dioxygenase. Maleylpyruvate can be by isomerization transformed into the fumarylpyruvate. Maleylpyruvate and fumarylpyruvate are by respec-

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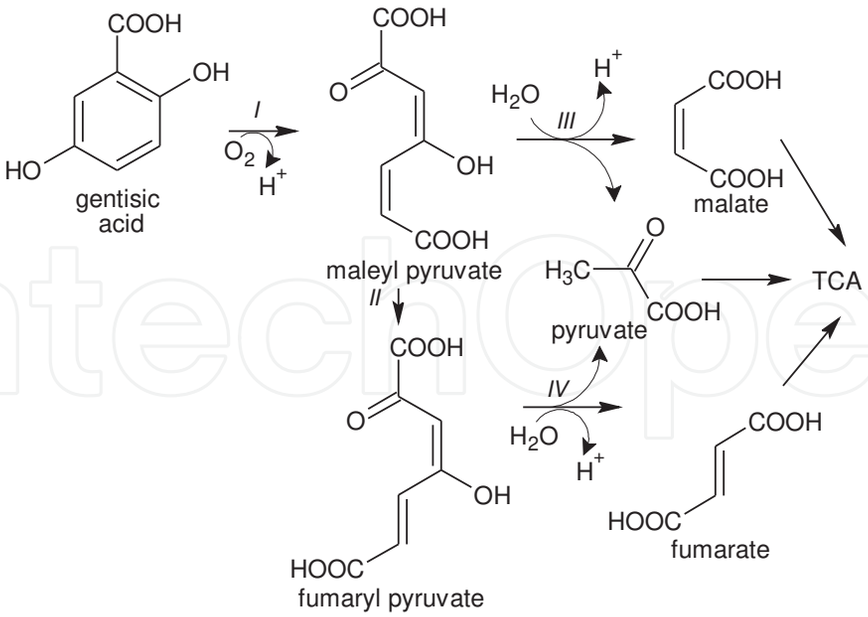

Figure 16. Gentisic acid degradation pathway [9]. *I* – gentisate-1,2-dioxygenase; *II* – maleyl pyruvate-*cis,trans*–isomerase; *III* – maleyl pyruvate-dehydrogenase; *IV* – fumaryl pyruvate-dehydrogenase; TCA – tricarboxylic acid cycle

tive hydrolase transformed into the malate or pyruvate, the tricarboxylic acid cycle intermediates [9].

2.2.6. Inhibition and activation of chlorobenzoic acids degradation

Due to the complexity of the described CBAs degradation pathways it can be expected that the CBAs degradation pathways will affect each other. This interaction can be positive as well as negative.

CBAs itself can affect microbial degradation of xenobiotics which are CBAs degradation products. For example, microbial degradation of PCBs can be by high CBAs concentration slowed down or even stopped [15, 44].

The influence of monochlorinated CBAs on the degradation of low chlorinated PCBs congeners by five different bacterial strains is already documented [15]. It was found that 3-CBA is the most potent inhibitor. The same result was obtained with monochlorinated PCBs degradation by strain *Pseudomonas stutzeri* [70], but in the case of microbial degradation of dichlorinated and trichlorinated PCBs was the inhibition effect of CBAs less significant. In the case of tests with *Pseudomonas testosteroni* B-356 and impact of monochlorinated CBAs on low chlorinated PCBs degradation, it was again found that the 3-CBA has the highest inhibition effect followed by 4-CBA and the least inhibition showed 2-CBA [16].

These findings can be explained by the formation of chlorocatechols in 3-CBA degradation with a higher probability than from other monochlorinated CBAs. Degradation of chlorocatechols in *meta*- cleavage pathway can lead to the reactive acylchloride formation. Acylchloride can inhibited 2,3-dihydroxybifenyl-1,2-dioxygenase from the upper cometabolic PCBs

degradation pathway (Figure 1.). How was proved by accumulation of hydroxylated biphenyls during PCBs degradation by strain *Pseudomonas testosteroni* B-356 [16].

Acylchloride can be also irreversibly bound on catechol or chlorocatechol-2,3-dioxygenase, the first enzyme in the *meta*- cleavage pathway. Its inactivation leads to catechols or chlorocatechols accumulation and therefore to the inhibition of CBAs degradation [71].

Another potentially dangerous intermediate is protoanemonine, substance with antibiotic properties. Protoanemonine is formed in the modified *ortho*- cleavage pathway from muconic or chloromuconic acid. The main effect of protoanemonine is growth inhibition of degrading microorganisms[72].

The presence of a mixture of CBAs can also cause inhibition or activation of CBA degradation. Strain *Burkholderia cepacia* JHR22 can degrade 2-CBA, 3-CBA, 4-CBA and 3,5-CBA [44] when present individually. This strain loses 2-CBA degradation ability when 2-CBA is present in mixture with 2,3-CBA or 3,4-CBA. On the other hand addition of 2,5-CBA or 2,6-CBA had no effect on 2-CBA degradation. When was 2-CBA added with 2,4-CBA strain *Burkholderia cepacia* JHR22 in addition to the 2-CBA degradation can cometabolically degrade 2,4-CBA.

Catechols or chlorocatechols are also potential inhibitors of bacterial CBAs degradation. If these intermediates of CBAs degradation are not enough quickly degraded they can be subject to the auto-oxidation or enzymatic polymerization with formation of brown or black pigment [73, 74]. The presence of this pigment inhibits CBAs degradation by affecting the shape of bacterial cells which may consequently lead to their death as in case of strain *Pseudomonas fluorescens* [75].

3. Phytoremediation

Together with the microorganisms participate on the elimination of xenobiotics green plants. Green plants use for the xenobiotics elimination four different strategies: extraction of contaminants from soil and water (mostly heavy metals), uptake and detoxication, in some cases even degradation (organic pollutants), volatilization (organic compounds and some metaloids Se and As) and stimulation of microbial degradation in rhizosphere or by endophytic microorganisms. All this processes are called phytoremediation (Figure 17.), the use of green plants for removal or transformation of pollutants from the environment [76, 77].

Phytoextraction is an accumulation of substances from the environment to the plant biomass. Precondition for the successful application of this process is that the plant is capable to take up contaminant by roots and ideally transport it to the aboveground parts, where contaminant is deposited in relatively high concentrations (more than 1 g per 1 kg of plant biomass). The disadvantage of this process is the possibility to re-release of contaminants from plant biomass, and their introduction into the food chain in the case of animals grazing on such plants [47, 76-78].

Phytovolatilization is uptake of pollutant by plant, which is followed by pollutant conversion to the volatile form and release in to the atmosphere [78, 79]. This type of phytoremediation is

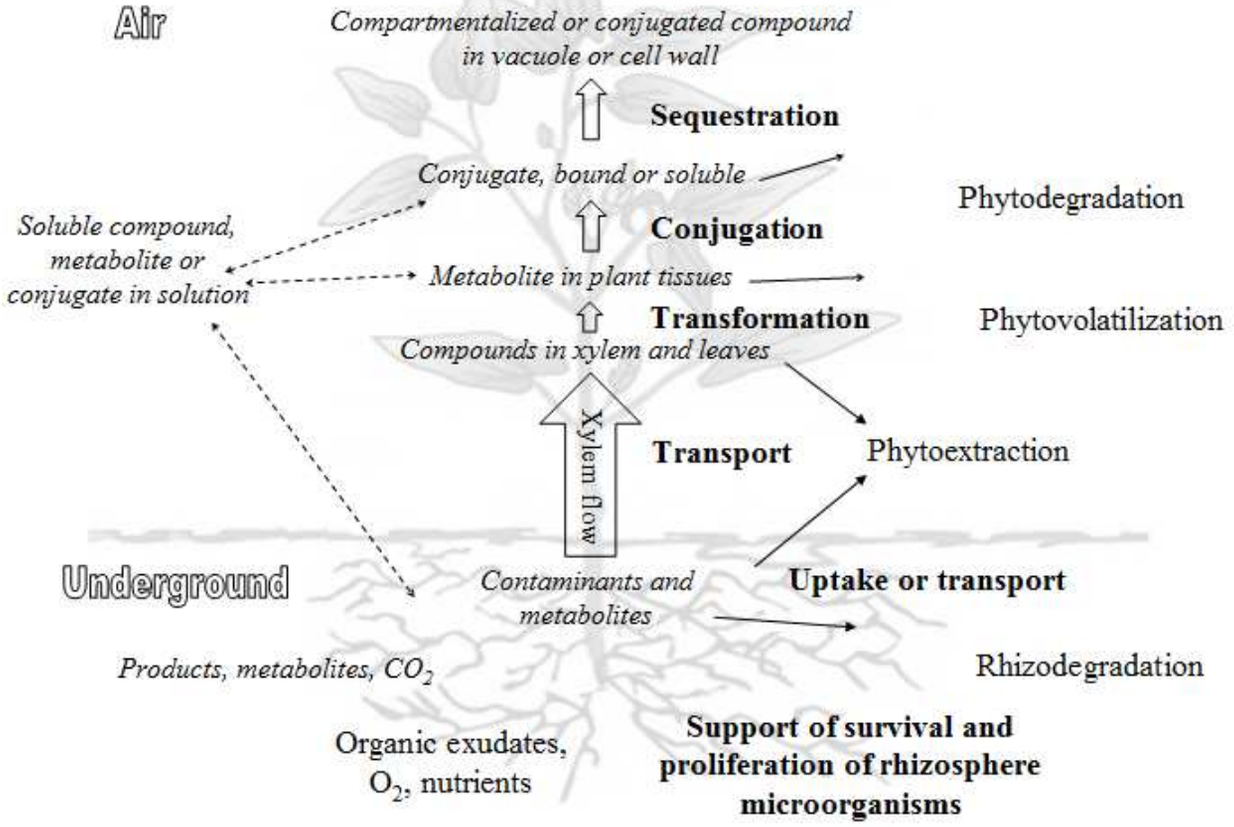

Figure 17. Schematic presentation of phytoremediation processes, adapted from [47].

controversial because it does not reduce contamination, only transfer pollutants from soil to the air [47, 76].

Rhizodegradation is stimulation of degrading microorganisms in plant rhizosphere. This stimulation can be done by support of proliferation or by creation of conditions suitable for better survival of microorganisms with degradation activity [47, 76].

Phytodegradation is a process when pollutants are taken up into the plant body, transformed and eliminated by plant metabolism. Phytodegradation can be considered also as a process in which contamination is reduced by enzymes released by the plant into the soil. Phytodegradation is most suitable for organic pollutants [47].

Plant metabolism of xenobiotics is in many aspects similar to the mammalian metabolism, therefore plants are called green liver of the planet. Uptake of xenobiotics is done by roots, from roots are then xenobiotics transported by xylem to the aboveground (harvestable) parts of plant, where xenobiotics are transformed to the non-phytotoxic metabolites. Metabolisation of xenobiotics in plant body is done in three phases. First is transformation followed by conjugation and the last phase is sequestration in plant tissues.

During transformation phase xenobiotics are subjected to enzymatic hydrolysis, oxidation or reduction. Reactive groups exposed or generated in transformation stage react with moieties such as glucuronate or glutathione in conjugation phase. Soluble conjugates are transported in the final stage to the vacuole or incorporated into the cell wall, thus preventing disturbing of the cell function by them [80]. Some water plants can transport conjugates outside the plant [47].

Using plants for decontamination has many advantages. It is a cost-saving process. After planting the area, are the costs for growing and harvesting biomass relatively low, and the obtained biomass can also be used to produce heat or electricity. It is aesthetically pleasing process positively accepted by the public. It causes minimal disruption to the environment. It is also applicable to the areas with trace or low pollution and is usable for a wide range of xenobiotics [77, 78, 81].

Phytoremediation has also drawbacks. The main disadvantage is that phytoremediation compared with physico-chemical methods is slower. It should take a few years to eliminate pollution and during the process most of the decontaminated areas can not be used for commercial purposes [78]. Therefore, the goal of the phytoremediation study is to increase the efficiency and speed of this process, by the understanding and improvement of the mechanism of phytoremediation and metabolic pathways involved in the conversion of xenobiotics by plants.

3.1. Plant metabolization of chlorobenzoic acids

About the CBAs metabolism in plants it is known, in comparison with microbial metabolism, very little. Although works have been published that deal with the CBA metabolisation in plants, it was not yet clearly demonstrated that plants degrade CBAs and not just accumulate CBAs in plant tissues.

Deavers et al. [82] investigated the metabolism of 4-CBA by cells of willow (*Salix viminalis*) under sterile conditions, as well as by whole plants. Cells were able to remove 65% of 4-CBA from the media during 360 hours with an initial concentration of 4-CBA 50 mg/l. For the whole willow plants, the concentration 50 mg/l of 4-CBA was toxic, and therefore the loss of 4-CBA was only in the range of 10-30%. When was initial concentration of 4-CBA reduced to 5 mg/l, willow plants managed during 305 hours to remove 70-90% of 4-CBA in dependence on the pH of the culture medium [82].

Other uses of the plants for metabolisation of CBAs has been described in plant tissue cultures of black nightshade (*Solanum nigrum*), tobacco (*Nicotiana tabacum*), horseradish (*Armoracia rusticana*) and alfalfa (*Medicago sativa*) [10]. In this case, the two initial concentrations were tested, 200 and 50 mg/l, and 11 different CBAs. From tested plant species the best ability to metabolize CBAs demonstrated black nightshade and horseradish. For *in vitro* grown cells of tobacco and alfalfa was the concentration of CBAs 200 mg/l toxic. So that named plant cells have not demonstrated metabolisation.

Tissue cultures of black nightshade and horseradish were within 14 days able to remove from the medium 90% of 2-CBA and about 30% of 2,3-CBA, 2,4-CBA and 2,5-CBA at an initial concentration of 200 mg/l, if the initial concentration was reduced to 50 mg/l this cell cultures

removed 100% of the mentioned CBAs. Culture of black nightshade with the same efficiency metabolized 3-CBA, 4-CBA and 2,3,5-CBA. Culture of horseradish metabolized other tested CBAs with 30-60% efficiency [10].

Siciliano and Germida [83] tested the ability of 16 different kinds of grasses grow in soil contaminated with 2-CBA (816 mg/kg). Only five of them had this ability, namely it were grasses *Bromus inermis*, *Agropyron intermedium*, *Bromus biebersteinii*, *Agropyron riparum* a *Elymus dauricus*. Only four last mentioned species were also able to metabolize 2-CBA from soil with efficiency from 32 to 42% in 60 days [83].

From these examples of CBAs metabolisation in plants is evident, that this process is not only influenced by the structure and concentration of CBA, but it is also influenced by plant species and plant capacity to "resist" the toxicity of CBA.

4. Plant and microbial cooperation on bioremediation

There are known several mechanisms for the promotion of the contamination removal from soil by plants. One of these mechanisms is a non-specific support of growth and proliferation of microbial communities by plants [84]. Support of microbial activity in the rhizosphere leading to the protection of plants from the effects of contamination and increasing the speed of xenobiotics elimination [85]. Another mechanism is specific support of degrading microorganisms in the rhizosphere of plants [86, 87], secretion of enzymes capable of catalyzing the conversion of contaminants in the soil by plants [88].

Plants can promote the growth of microorganisms in the rhizosphere by excretion of root exudates into the soil [89]. Exudates may contain various ions, free oxygen, water, enzymes (e.g. laccase, peroxidase, dehalogenase, nitroreductase), substances of the saccharide nature to facilitate root growth and diverse mixes of primary and secondary metabolites. Organic compounds in exudates are divided into two groups. One is for substances with low molecular weights such as amino acids, organic acids, sugars, phenolic compounds and other secondary metabolites and second is for substances of higher molecular weight such as proteins and polysaccharides [90].

The problem of the use of the cooperation in bioremediation is that the increase in the number of microorganisms in the rhizosphere of plants does not always mean increased degradation of the contamination. An example of this behavior is experiment when exudates of perennial ryegrass (*Lolium Perence*) were used in the degradation of phenanthrene [89]. Results showed that despite the increased number of microorganisms caused by addition of exudates, no increased phenanthrene degradation was observed. Phenanthrene degradation in this case was even half than in the control without exudates.

Just as plants can affect the growth of microorganisms, microorganisms can also affect the growth and survival of plants in the contaminated areas. Microorganisms can help plants by production of protective biofilms or antibiotics acting as a protection against organisms potentially pathogenic for plants [91, 92]. In addition, the activity of certain microorganisms

is source of nutrients for plant e.g. nitrogen compounds. Microorganisms may also increase the solubility of minerals such as phosphorus, and thus make them more accessible for plants. Another mechanism of protection of plants by microorganisms is the synthesis of low molecular weight siderophores in addition to phytosiderophores. Siderophores bind the free iron in the rhizosphere of plants. Lack of free iron negatively affects the proliferation of fungal pathogens in the root system of plants [93, 94].The presence of microorganisms can also initiate the synthesis of plant hormone such as auxins, cytokinins and gibberellins, which promote the growth of plants [91, 95]. Equally important is the mechanism for reducing stress by reduction of stress hormone ethylene concentration in plant by synthesis of microbial enzyme 1-aminocyklopropan-1-carboxylate deaminase (ACC deaminase) [96].

During the life of the plant its growth may be influenced by microorganisms with one or more of these mechanisms. Influence is most evident in plants grown under stressful conditions.

In a consortium consisting of microorganisms and plants growing in the contaminated soil, in addition to the above mentioned mechanisms, can occur production of metabolites or intermediates of degradation no matter whether by plant or microorganism. These metabolites may be by the other partner further degraded and metabolized or they may be toxic. The study of the metabolism of both biological systems and their possible influence should lead to the obtaining of such a system consisting of plant - microorganisms, which increases speed and efficiency of the xenobiotics degradation process.

4.1. Plant and microbial cooperation on chlorobenzoic acids remediation

The issue of co-operation of plants and microorganisms on degradation of CBA is solved in several publications.

Haby and Crowley [84] compared the degradation capacity of soil from the rhizosphere of perennial ryegrass (*Lolium perenne*) with non-vegetated soil. Results showed that the degradation rate of 3-CBA was at the beginning of the experiment accelerated by the rhizosphere soil, they also showed that in soil from the rhizosphere is increased amount of microorganisms [84].

Dittmann et al. [97] examined the ability of three months old pine seedlings (*Pinus sylvestris*) with mycorrhizal fungi *Suillus bovinus* to degrade 3-CBA. They found that 3-CBA is accumulated in the aboveground parts of the seedlings and removal efficiency of 3-CBA from the soil by a consortium was very low (around 25% for 4 weeks). In plant tissues were not detected elevated concentrations of Cl^- in comparisom with control plants grown in uncontaminated soil. Therefore they concluded that 3-CBA was not or only to a very small extent degraded by plants [97].

The aforementioned grass *Elimus dauricus*, together with microorganismus *Pseudomonas aeruginosa* R75 and *Pseudomonas savastanoi* CB35 was able to remove almost 50% of 2-CBA from soil during 56 days [98]. The consortium showed greater metabolic efficiency than non-vegetated soil.

In this work has been continued testing the degradation capacity of microorganisms and plants with other grasses and other CBAs or with a mixture of CBAs. The same composition of the

consortium, thus *Elimus dauricus* and strains of *Pseudomonas aeruginosa* R75 and *Pseudomonas savastanoi* CB35 was able to remove 74% of 3-CBA from the soil. *Bromus biebersteinii* with strain *Alcaligenes* sp. BR60 remove 56% of 2,3-CBA from the soil and the same consortium was able to metabolize 61% of 2,3-CBA and 50% of 3-CBA if both CBAs were added together. A consortium consisting of *Elymus angitus* and strains *Pseudomonas aeruginosa* R75 and *Pseudomonas savastanoi* CB35 eliminate 46% of 2,5-CBA from soil and from the mixture of 3-CBA, 2,3-CBA and 2,5-CBA this consortium has removed around 40% of all three CBAs from soil [99]. These results were obtained by testing a large number of combinations of grasses with bacterial inoculants. Most of the combinations did not show the positive impact on reducing the tested CBA or a mixture of CBAs from soil. Inoculants did not reduce CBA phytotoxicity and caused plant death in contaminated soil.

The above examples show that the use of plants and microorganisms in bioremediation technologies has great potential. However, selection of suitable pair of plant and microorganism is very complicated. We can assume that not only depend on the type of contamination, but it requires a deeper understanding of the principles of cooperation between this two organisms.

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