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Chapter 5

The Adaptation Mechanisms of Bacteria Applied in Bioremediation of Hydrophobic Toxic Environmental Pollutants: How Indigenous and Introduced Bacteria Can Respond to Persistent Organic Pollutants-Induced Stress?

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Abstract

The chapter describes the aspects of bioremediation that are related to survival and metabolism of bacterial degraders in the adverse environment contaminated with dangerous hydrophobic chemicals, polychlorinated biphenyls (PCBs). Successful environment decontamination requires bacterial strains that possess appropriate enzymes and are able to degrade particular contaminants. This chapter deals mainly with the adaptation mechanisms that allow bacteria to decrease toxic effects of the dangerous compounds on cytoplasmic membrane as the first contact point of pollutants and the bacterial cell. Many responses have been observed in bacteria that counteract the effects of toxic environmental organic pollutants: saturation-rigidification of cell membrane, *cis*/*trans* isomerization of fatty acids, increased content of cyclopropane fatty acids, and changes in branched fatty acids and cardiolipin, production of stress proteins, and elimination of toxic compounds using efflux pump. The study of these mechanisms is the first step in selection of appropriate resistant bacterial strains for bioremediation applications. Next steps should include study of degradation potential and efficacy of the most resistant strains. Setting up suitable experimental systems to examine the cell responses to toxic environmental organic pollutants in the adverse environment and optimal conditions for metabolism of bacterial degraders are important issues in the current bioremediation research agenda.

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1. Introduction

Due to more than 200 years of industrialization and to the use of dangerous substances in many production processes, the countries across the world are facing the problem of soil, sediment, and water matrices contamination. Contaminated sites, namely environmental burdens, generally resulted in past and also arise nowadays from the manufacturing, storage, use, and disposal of hazardous chemicals and materials. It is now widely recognized that polluted sites pose threats to human health and the environment.

Polychlorinated biphenyls (PCBs) represent an environmental concern due to their hydrophobicity and toxicity. Although the production of PCBs has been banned and their use heavily restricted, they still pose an environmental problem due to their presence in old electrical transformers, capacitors, landfills, and in contaminated soil and sediments mainly in the areas around the former production facilities [1, 2]. Their physical and chemical properties such as thermal and chemical stability, resistance to degradation, and general inertness contribute to their persistence in the environment [3]. PCBs represent potential health risks for living organisms due to their lipophilic nature, bioaccumulation, and potential carcinogenic properties [4]. Hydroxylated PCBs (HPCBs), known PCB metabolites, have been detected in human serum samples and wildlife blood samples [5]. Numerous adverse health effects in human have been associated with these compounds. HPCBs are capable of mimicking a thyroid hormone, thyroxin [6], and may generate reactive oxidative species and cause DNA damage. Studies performed with the individual PCB congeners show that PCB toxicity and biodegradability are structure related as well [7].

Many conventional and sustainable remediation techniques have been invented to destroy hazardous organic pollutants [8]. The finding that both Gram-negative bacteria, such as *Achromobacter*, *Alcaligenes*, *Burkholderia*, *Commamonas*, *Pseudomonas*, and Gram-positive bacteria, such as *Bacillus*, *Corynebacterium*, and *Rhodococcus*, are able to degrade some PCB congeners opened the door to implement biological technologies. Bioremediation technologies using degradation capacity of microorganisms, mainly bacteria, have been seen ecological and economical alternative approach to physicochemical processes to eliminate diffusive contamination of persistent organic pollutants (POPs) in various environmental matrices, e.g., soil, sediments, and sludges. Bioremediation is an attractive, generally low-cost, innovative technology that is a sustainable approach to clean up organic compounds from contaminated areas. Bioremediation represents a perspective and prospective technique for treatment of polluted environments which involves usage of microorganisms and/or plants for pollutant biodegradation or biotransformation. The technology can be performed as natural attenuation or employed as an assisted bioremediation: biostimulation (addition of nutrients and inducers to fortify and stimulate the growth and metabolism of indigenous microorganisms), and bioaugmentation (introduction of indigenous or suitable exogenous bacteria to enhance

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biodegradation of relevant pollutant) [9–13]. However, successful soil bioaugmentation requires not only application of the individual bacterial strain or a bacterial consortium with the required degradation ability but also of the microorganisms able to survive in the adverse environment [14–17]. Poor survival of the inoculated microorganisms (usually bacteria) and low bioavailability of the hydrophobic carbon source are usually the main obstacles to the successful inoculum amendment. Moreover, the bottleneck for the successful catabolism of a recalcitrant hydrophobic compound is most often not the nature of the biochemical pathway for its degradation, but the overcoming of the endogenous and exogenous stress associated to the utilizing conditions. Although many bacteria have ability to metabolize, e.g., PCBs, high concentrations of these chemicals act as environmental stress factor and inhibit cell survival and then ability to metabolize these pollutants. If bacterial strains wanted to survive, they had to develop efficient adaptation mechanisms in the adverse environment [18, 19].

For the purpose to select the degradation-effective and adverse environment-resistant bacterial strain from 11 environmental isolates, obtained from the PCB-historically contaminated sediment and identified using molecular-biological methods [20], our research was focused on the study of adaptation mechanisms and responses of bioaugmented bacteria during the biological treatment of water and sediment matrices contaminated with PCBs. Since PCBs are highly hydrophobic, they may efficiently cross cell membrane through free diffusion. The effects of PCBs, chlorobenzoic acids (CBAs, PCB-biodegradation end products), biphenyl, and terpenes (the potential inducers of PCB degradation) on bacterial cytoplasmic membrane were determined [10, 11, 15, 19]. Only the resistant bacteria that possess the appropriate enzymes may play a major role in bioremediation technologies.

2. Response mechanisms of bacterial cells to adverse environment

2.1. Saturation of membrane fatty acids

The most adaptive mechanisms are concerned with maintenance of the cell membrane fluidity and lipid-phase stability [21]. Fluidity of cytoplasmic membrane is a very important characteristic of the membrane structure and is defined as the reciprocal value of its viscosity. It can be modulated by the alteration of fatty acids that build membrane phospholipids. Extreme environmental conditions activate in cells a series of processes that allow microorganisms to minimize their negative impact. Bacteria have developed various mechanisms to eliminate toxic compounds present in the environment. Being at the interface between the cell and the environment, the cytoplasmic membrane is the first site of contact between the cell and contaminant. Hydrophobic organic pollutants change the fluidity of bacterial membrane that can lead to a significant disturbance of physiological function and apoptosis. This is the reason why membrane flexibility and adaptation ability largely determine the survival of the cell [22, 23]. Since fatty acids are the major constituents of membrane phospholipids, modulation of number and position of double bonds of acyl chains by specific fatty acid desaturases plays crucial role in preserving a suitable dynamic state of the bilayer during environmental impact [24]. One of the observed membrane adaptation mechanisms is the increase of saturation of bacterial membrane lipids. The linear acyl chains of saturated fatty acids can be tightly packed

leading to lower fluidity (**Figure 1**) that counteracts the fluidizing effects caused by the presence of toxic organic compounds [25]. Although bacterial cell tries to increase membrane rigidity to counteract the effects of organic pollutants, the membrane must be able to perform its physiological functions. Therefore, a part of membrane must stay in liquid-crystalline phase. The mechanism of increase of saturation degree has limitation due to the condition of synthesis of saturated fatty acids. In bacteria, only the energy-dependent *de novo* biosynthesis of saturated fatty acids allows the increase in the degree of saturation, which may also be the reason why alteration in the degree of saturation was only observed in growing cells [26, 27]. Therefore, under growth-inhibiting conditions, lipid biosynthesis is stopped due to stringent response regulation, and that is why, only growing cells can perform such kind of membrane adaptation [25]. A correlation between an increase in the degree of saturation of membrane fatty acids and increased tolerance toward the toxic compounds in *Pseudomonas putida* P8 was described [28]. This phenomenon is thought to be one of the major long-time adaptive mechanisms in microorganisms exposed to toxic aromatic compounds. Due to this, the bacterial membranes become more resistant to the fluidizing action of aromatic compounds, which allows the cells to survive in hydrocarbon-contaminated sites [14, 29].

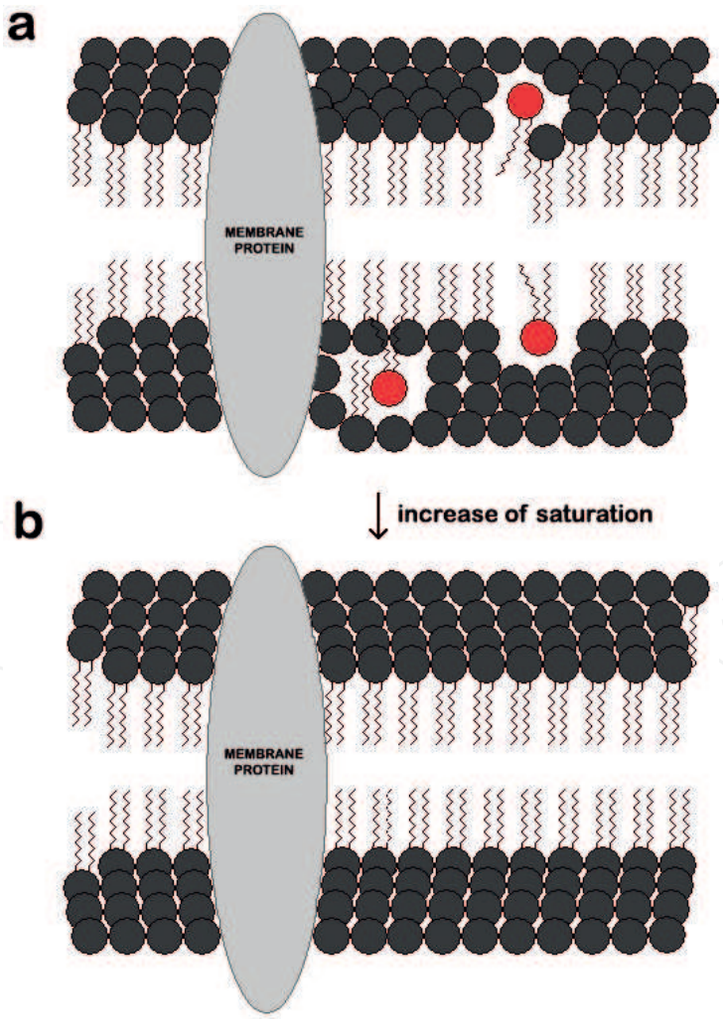


Figure 1. Increase of the synthesis of saturated fatty acids (grey circles) instead of unsaturated fatty acids (red circles) leads to the higher membrane saturation, higher rigidity, and lower fluidity. Modified according to [16].

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2.1.1. Cis/trans isomerization of unsaturated fatty acids (UFAs)

Various bacterial strains, e.g., *Pseudomonas*, can adapt to the presence of toxic compounds and their fluidizing properties by isomerization of *cis* unsaturated fatty acids to their appropriate *trans* isomers. These two forms of unsaturated fatty acids have different steric structure. The *cis* configuration of the acyl-chain has a nonmovable bend of 30°, which causes steric hindrance and disturbs the highly ordered fatty acid package [30]. In contrast, the steric behaviour of *trans* fatty acids and saturated fatty acids is very similar. Nonmovable bends of *trans* fatty acids have 6°. Both *trans* and long chain saturated fatty acids possess a long-extended conformation. It enables them to adopt a denser packing in the cytoplasmic membrane and allows protecting membrane against the fluidizing molecules. That is the reason why the transformation of *cis* to *trans* fatty acid leads to the decrease of membrane fluidity (**Figure 2**). Another reason for an ordered packing of *trans* fatty acids compared to *cis* isomers is their higher T_M (transition temperature). This mechanism was monitored in growing as well as in growth-inhibiting conditions. *Cis-trans* isomerase is constitutively present, does not require ATP or other cofactors including NAD(P)H and glutathione, and works in the absence of *de novo* synthesis of lipids. The *trans* fatty acids are formed by direct isomerization of the

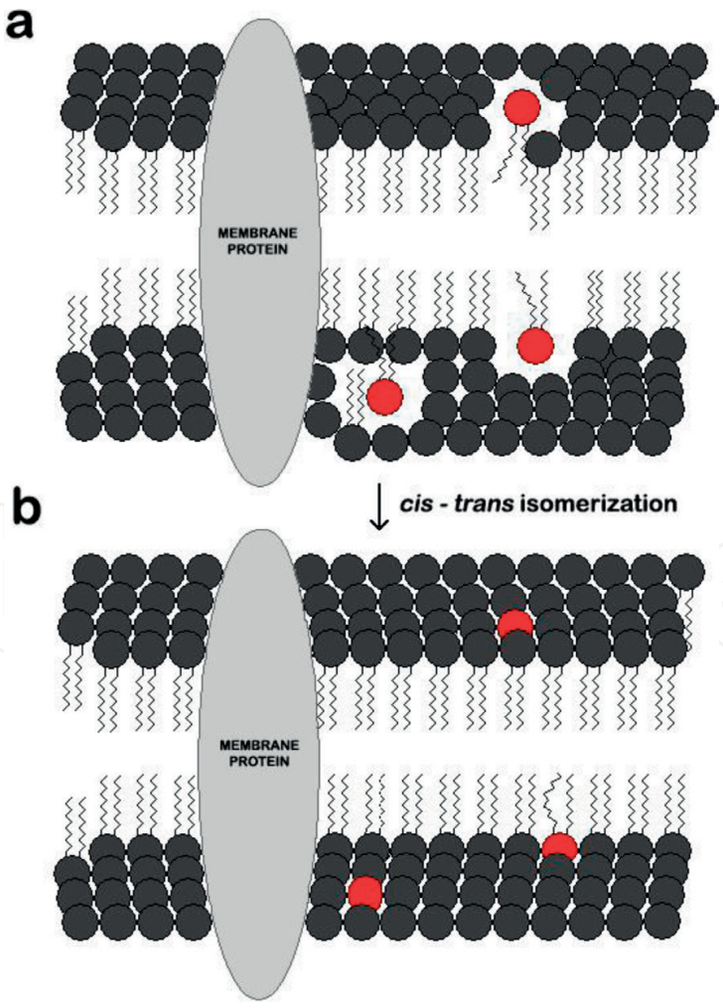


Figure 2. The transformation of *cis* unsaturated fatty acids into corresponding *trans* isomers decreases the membrane fluidity of bacterial cell. Modified according to [16].

complementary *cis* configuration of the double bond without a shift position. Because of the steric differences between *cis* and *trans* configurations, this conversion reduces membrane fluidity and counteracts against the stress [31].

2.1.2. Changes in cyclopropane and branched fatty acids: anteiso-/iso-branching

Changes in cyclopropane and branched fatty acids can be observed in the adverse environment as well. Higher concentration of organic pollutants stimulated production of cyclopropane fatty acids in some bacterial strains [19, 22, 29]. The role of these fatty acids is still not understood in detail. Some authors indicated that cyclopropane fatty acid formation is one of the most important mechanisms that protect bacterial cells against many chemicals [23]. In the presence of toxic compounds or toxic conditions, bacteria increase the production of *iso*-branched fatty acids on the expense of *anteiso-* forms to decrease the membrane fluidity [9, 14, 32]. Transition temperatures of the branched fatty acids are lower for the *anteiso*-fatty acids. This difference together with steric differences causes a remarkable change in the fluidity of the membrane when the species of branched fatty acids are changed from *iso-* to *anteiso*-form. The effect on transition temperature caused by a change from *anteiso-* to *iso*-branching in G^+ bacteria is comparable to the isomerization of *cis* to *trans* unsaturated fatty acids in G^- bacteria. Even the volume occupied with *anteiso*-fatty acids is higher than that occupied with *iso*-FAs. According to the different physicochemical properties of those two species of branched FAs, the bacteria showed a decreased amount of *anteiso*-FAs when grown under adverse conditions to decrease the fluidity of membrane and diminish incorporation of the pollutants into membrane structures [14, 16].

2.2. Changes in phospholipids

Bacteria contain several different phospholipid headgroups in their cytoplasmic membrane. Each of them holds specific function to maintain cell vitality. In the presence of environmental perturbations, cells alter the amount of phospholipids. Changes in phospholipid headgroups on environmental pollution are rarely studied than fatty acid alteration. Weber and de Bont [33] studied the effects of the composition of the phospholipid headgroups on the membrane fluidity. Phosphatidylethanolamine (PE) is the most abundant phospholipid in bacterial membrane that comprises more than 70% of all phospholipids [27]. It provides lateral pressure to bacterial membrane bilayer and keeps the position of amino acids. It is a nonbilayer forming lipid because of its steric conformation (small glycerol group and high acyl-chain volume). Nonbilayer aggregates (preferred hexagonal conformation) of cytoplasmic membrane are important in cell division, membrane fusion, and in the lateral proteins and lipid motion. The ratio between bilayer and nonbilayer forming lipids varies in response to environmental changes. Organic solvents like benzene and toluene can reduce the transition temperature of membrane lamellar gel to liquid-crystalline phase (T_M) and enhance the formation of nonbilayer aggregates with decreasing the transition temperature from cylindrical into inverted hexagonal phase (T_{LH}). Stabilization of the T_M is important to sustain membrane fluidity and stability. T_M of cytoplasmic membrane can be slightly modified by membrane phospholipids (each of them has different T_M), which can affect bilayer stability of membrane. Cultivation of

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Pseudomonas putida S-12 with toluene decreased the amount of PE and increased the content of phosphatidylglycerol (PG) and cardiolipin (CL). This alteration could stabilize membrane by lowering the fluidity. However, phospholipids have much higher effects on bilayer stability (T_{LH}) than on membrane fluidity (T_M) because of their ability to form hexagonal or lamellar structures [33]. Based on these facts, the decrease of PE content leads to higher bilayer stability. Nevertheless, bacterial cell tries to keep balance between bilayer and nonbilayer phospholipids to maintain its physiological function. Donato et al. [34] described the effects of DDT on the bacterial strain *Bacillus stearothermophilus*. This compound induced a very significant increase of the PE membrane content with a parallel decrease of PG content. This alteration was accompanied by an increase of straight chains and a parallel decrease of branched fatty acids in cytoplasmic membrane. DDT promoted more ordered membrane with an increase of the T_M temperature to higher values that led to higher membrane rigidity. However, increase in PE and decrease of PG amounts is not a usual response of the bacteria. PG is important in CL synthesis and plays a role in protein translocation across the membrane [35].

Based on their polarity, toxic organic solvents can accumulate in different membrane sites. This affects their ability to change the membrane bilayer stability by formation of an inverted cone (polar pollutants) or cone structures (nonpolar pollutants). Polar pollutants as ethanol can accumulate between the glycerol headgroups. This process can destabilize bilayer-nonbilayer balance. Bacterial cells react to these effects by the formation of a lipid with a small headgroup volume (e.g., monoglucosyldiglyceride). The presence of benzene increases the formation of hexagonal aggregates. Cells counteract this phenomenon by stimulation of production of lamellar phospholipids (e.g., diglucosylglyceride). Similar effects can be observed in the presence of toluene. Toluene can incorporate into the membrane between the acyl chains. The cell responds by production of the higher amount of CL to stabilize the bilayer. CL has a larger headgroup volume compared to PE. The decrease of PE production and increase of CL content will increase the volume of headgroups. This can compensate toluene-induced increase of acyl chain volume and stabilize the bilayer. Moreover, CL has 10 K higher T_M than PE. Due to this fact, CL increases the membrane rigidity, while toluene induces disordering of acyl chains. Some opposite effects occur in the presence of polar ethanol [33, 36]. The regulation of phospholipid headgroups controls the ratio between bilayer and nonbilayer membrane structures and the bilayer surface charge density.

2.2.1. Adaptation responses of bioaugmented bacteria used in biological treatment of contaminated water and sediment matrices to nonpolar PCBs and polar 3-CBA

The effects of nonpolar PCBs and polar 3-chlorobenzoic acid (3-CBA, one of PCB-degradation end product) were assessed in our laboratory using four bacterial isolates obtained from the long-term PCB-exposed contaminated sediment (*Ochrobactrum anthropi* and *Pseudomonas veronii*) and soil (*Alcaligenes xylosoxidans* and *Pseudomonas stutzeri*) [37]. About 100 mg L^{-1} of each pollutant was added separately into the minimal mineral media at the beginning of cultivation together with the bacterial inoculum (1 g L^{-1}). Adaptation responses in phospholipid headgroups were analyzed after 6 days of cultivation on the rotary shaker (180 rpm) at 28°C in the dark (**Figure 3**). The differences in adaptation responses toward polar and nonpolar

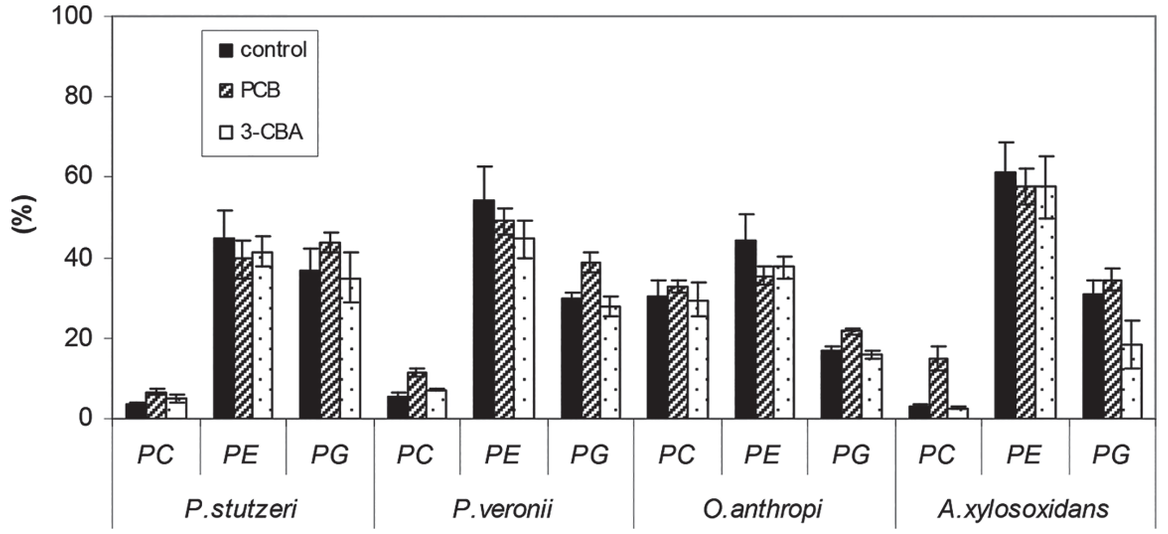


Figure 3. Percentage amount of membrane phospholipids after the addition of nonpolar (PCBs) and polar (3-CBA) toxic pollutants into the minimal mineral medium in the presence of two bacterial strains isolated from a long-term PCB-contaminated soil—*Pseudomonas stutzeri* and *Alcaligenes xylosoxidans, and* two bacterial strains isolated from a long-term PCB-contaminated sediment—*Pseudomonas veronii* and *Ochrobactrum anthropi.* Modified according to [16]. PC, phosphatidylcholine; PE, phosphatidylethanolamine; and PG, phosphatidylglycerol.

contaminants can be seen on the examples of PC and PG. Only a minority of bacterial strains contain PC in their membrane [16]. This phospholipid belongs to a bilayer-forming group similarly to PG [38]. An increase in PC accumulation in membrane was observed after addition of nonpolar PCBs. Polar 3-CBA did not rapidly affect the amount of this phospholipid in the membrane. Only slight increase of PC content was observed in both *Pseudomonas* species after 3-CBA addition. On the contrary, both pollutants caused the decrease of PE amount in all studied strains. As mentioned in previous part, PE belongs to nonbilayer phospholipids. The presence of both toxic pollutants leads to their accumulation in membrane and destabilizes the bilayer conformation. Cells counteract these effects by reducing the nonbilayer phospholipid fraction to increase the membrane stability. This phenomenon was accompanied by an increase in membrane saturation and *cis*/*trans* isomerization to decrease membrane fluidity [19]. Nonpolar compounds are able to accumulate between the acyl chains of phospholipids and stimulate the hexagonal formation and increase T_M. Because of such accumulation, increase of PG content in membrane can be expected [33]. Our results obtained using the PCBs are in accordance with this assumption. The presence of 3-CBA caused the decrease of PG content. This can be explained by the ability of a polar compound to accumulate between the polar phospholipid parts (glycerol headgroups) and by a stimulation of micellar formation (interdigitated phase). PG has a larger headgroup volume; therefore, a decrease of this membrane component increases membrane stability. The addition of PCBs evoked increase of PG and PC membrane incorporation and decrease of PE in bacterial cells. These results are in agreement with the results obtained with other nonpolar toxic compounds [33].

The effects of toxic environment were not confirmed in the case of addition of 3-CBA at the time of inoculation in both strains of *Pseudomonas* genera. We assumed that 3-CBA is extremely toxic when present at the lag phase of the bacterial cell growth. The adaptation mechanisms

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occurred in the cytoplasmic membrane (increase of *trans*/*cis* ratio) were not efficient enough to counteract the effects of 3-CBA (**Figure 4a**). Such toxic conditions are responsible for the disability of both *Pseudomonas* strains to adapt to polar organic acid. Therefore, a biomass amount decreased below the inoculation amount. The determination of branched fatty acids was performed because of their ability to change membrane fluidity (**Figure 4b**). *Anteiso-*/*iso-* ratio reflected the changes in branched fatty acids. These fatty acids are generally produced to increase the membrane fluidity. *Anteiso-*(3-methyl) fatty acids exert stronger influence on membrane fluidity than *iso-*(2-methyl) isomers due to their steric configuration and different transition temperatures. Under the toxic condition, bacteria increase the production of *iso-*fatty acids on the expense of *anteiso-* forms to decrease the membrane fluidity [9, 14, 32]. The 3-CBA addition to the 3-day-old cultures revealed enhancement of the adaptation mechanism compared to the addition of PCBs with all strains except for *A. xylosoxidans*. Interesting information was observed in branched membrane fatty acids in both studied *Pseudomonas* strains. The amount of these acids increased when the toxic compounds caused a growth inhibition of *P. stutzeri.* This effect was also observed in *P. veronii* after the addition of organic pollutants and in absence of *cis* to *trans* isomerization. Because of lower production of unsaturated fatty acids under these conditions, cell may try to maintain liquid-crystalline phase of at least part of membrane with these fatty acids [33]. *A. xylosoxidans* and *O. anthropi* were confirmed as most adapted to tested chloroaromatics among all studied bacterial strains [37]. Thus, *A. xylosoxidans* and *O. anthropi*, bacterial strains isolated from different contaminated matrices, soil, and sediment, both long-time polluted with PCBs, could be useful in further bioremediation studies.

2.2.2. Increase of phospholipid amount

A unique phospholipid that plays an important role in cell membrane adaptation is cardiolipin (CL). Increase in its synthesis strongly enhances the adaptation ability of bacterial cell to the presence of organic solvents. This mechanism was observed mainly in *Pseudomonas* species [39]. Together with PG, it represents the most abundant anionic lipid component of bacterial membrane. This phospholipid is markedly present in many of G^+ bacteria. It may trap protons in an acid structure and bind to many of unrelated proteins. The molecule consists of two phosphatidic acid residues linked by a glycerol. It contains four fatty acid chains per molecule and possesses one negative charge per headgroup. CL is synthesized with cardiolipin synthase in the cytoplasmic membrane. The synthase catalyzes the transfer of phosphatidyl group between two phosphatidylglycerol molecules. This enzyme reacts with two PG molecules, one acting as phosphatidyl donor and the other as phosphatidyl acceptor. This enzyme does not have strict substrate specificity and may act in the reverse direction and decompose CL. Trace amount of CL occurs in bacterial cells during the exponential growth phase. Accumulation of CL increases at the beginning of stationary phase. It is the most stable of all membrane phospholipids and is essential for the survival upon long-time starvation. Only *de novo* synthesis of CL was described in bacteria [40]. Prokaryotes can change the amount of this lipid depending on their physiological status and growth conditions. Increase of the amount of CL is a known adaptation mechanism in the stress environment. It may reflect a requirement for enhancement of the structural integrity of the cytoplasmic membrane or for the support of stress-related increases in energy transduction [41]. CL stimulates changes in the physical properties

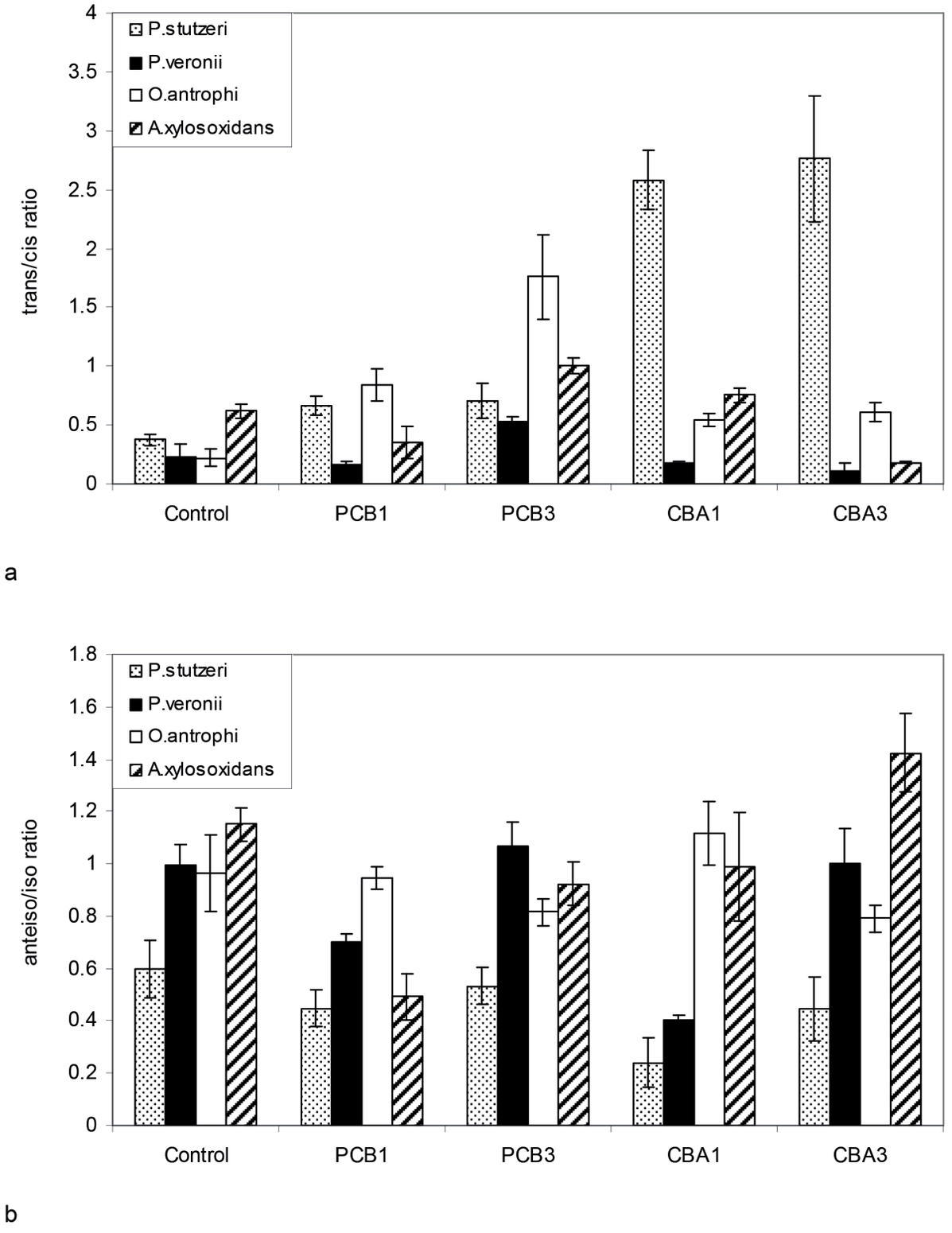


Figure 4. (a) The *trans*/*cis* ratio and (b) the *anteiso*/*iso* ratio of phosphatidylethanolamine and in control experiment (without PCBs). PCB1, PCBs added at the first day of cultivation; PCB3, PCBs added to the 3-day-old culture; CBA1, 3CBA added at the first day of cultivation; and CBA3, 3-CBA added to the 3-day-old culture. Modified according to [37].

of cytoplasmic membrane. Even small amounts of CL decrease the lateral interaction within the monolayer leaflet, which decreases the energy required to stretch the membrane and could favor the creation of membrane folds [42]. This is the reason why CL is concentrated

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in polar and septal regions of the cell. It can form nonlamellar structures that are required for membrane curvature and lead to the formation of clusters. The advantage of its unique conformation enables the nonlamellar structure to pack tightly forming microdomains which are stabilized by membrane proteins [43, 44]. Recent studies confirmed that bacteria with cardiolipin synthase deficiency are more vulnerable to organic solvents [45]. The mutant bacterium that is not able to synthesize CL was used to find out whether the *cis*/*trans* isomerase is able to compensate CL in adaptation mechanisms. The mutant was not able to grow, which indicates that *cis*/*trans* isomerase was not fully able to replace adaptation effects of CL [46].

2.3. Toxic pollutants as substrates for the efflux system

Such elimination of unwanted chemicals takes place by an uncontrolled efflux and accelerates active extrusion of structurally unrelated compounds from the cytoplasm or the cytoplasmic membrane to the external space. Toxic organic pollutants may represent substrates for the efflux system. Several studies indicated the importance of physical properties of compounds (hydrophobicity and molecule charge) for the determination of specificities of this mechanism [47–49]. The efflux system transporters for organic compounds identified in multidrug resistant G− bacteria belong to the resistance-nodulation-cell division family (RND) of pumps that are encoded chromosomally [50]. This system consists of complex transporters, which export toxic compounds through the cell membranes in a single-energy-coupled step. It requires a cytoplasmic membrane export system, which acts as an energy-dependent extrusion pump, a membrane fusion protein, and an outer membrane factor [51]. It was found that primary multidrug efflux system AcrAB-TolC facilitated the efflux of hydroxyl-PCBs out of the cells [46]. These multidrug-resistant pumps may affect the accumulation and degradation of PCBs by bacteria. Moreover, adapted bacteria of *Pseudomonas* sp. accumulated lower amount of trichlorobenzene in cells than nonadapted strains [39]. Similar results were published with toluene [51]. The ability of *E. coli* to eliminate PCBs and hydroxyl-PCBs was studied by Geng et al. [52]. The primary efflux system facilitated the elimination of hydroxylated PCBs (HPCBs) out of the cell. Since AcrAB-TolC is constitutively expressed in *E. coli* and is conserved in all sequenced Gram-negative bacterial genomes, the results suggest that the efflux activities of multidrug-resistant pumps may affect the cellular accumulation and degradation of PCBs in G^- bacteria. The multidrug resistance and the efflux of toxic pollutant by *P. aeruginosa* were determined [53]. Some of efflux pumps act on a restricted range of substrates. An example of such pump is TtfDEF pump from *P. putida* DOT-T1E, which extrudes only toluene and styrene [54]. Other pumps have a broad range of structurally diverse compounds. MexAB-OprM from *P. aeruginosa* can extrude hexane, xylene, and PCBs [48].

2.4. Production of stress proteins

Other known response of bacterial cells to POPs presence is the production and overexpression of stress proteins [55–59]. The production of shock proteins belongs to nonspecific general stress responses. Induction of stress proteins in *E. coli* with benzoate has been reported [60]. Other stress protein is induced by 4-chlorobiphenyl and biphenyl in *B. xenovorans* LB400 [55]. Expression regulation of the stress proteins was reviewed and the role of alternative sigma factor σB in this adaptation was emphasized [62]. This factor controls the production of bmrUR operon in *Bacillus subtilis* necessary for production of multidrug efflux proteins [63]. Toxic environment

acts not only on the envelope but usually affects the cell proteome as well. Damaged proteins can be replaced with the newly synthesized. However, this method is not efficient under nutrient limitations. Therefore, the proteome repair is required to maintain cell viability.

Three major mechanisms operate in bacteria after a proteome damage induced by adverse environment [64]. First mechanism includes the chaperones, which assist in proper *de novo* folding of proteins and provide an important means of restoring activity to damaged proteins. Second mechanism describes the existence of enzymatic repair systems that directly reverse certain forms of protein damage, including proline isomerization, methionine oxidation, and the formation of *iso*-aspartyl residues. Third mechanism concerns proteolysis of abnormal proteins, which cannot be repaired. No effect on membrane lipids of *B. xenovorans* LB400 in the presence of 4-CBA and 2-CBA was observed. The primary adaptation was revealed as an overexpression of proteins (mainly the overproduction of catechol-1,2-dioxygenase, belonging to 3-oxoadipate chlorobenzoate degradation pathway). Stress proteins, metabolic proteins, and elongation factors were stimulated as well [56].

2.5. Changes in bacterial cell morphology as a stress response

Cell envelope of microorganisms consists of cell wall and cytoplasmic membrane. These covering compartments protect cell nucleus against outside effects and help in communication with other cells. Most of adaptation mechanisms relate to cytoplasmic membrane as highly selective barrier. Moreover, the first line of cell protection is based on the alteration of the membrane composition that leads to lower fluidity and permeability toward toxic compounds. The surface structure is quite dissimilar in G^+ and G^- bacteria. G^+ bacterial strains have thick murein-containing cell wall convoluted with teichoic acids. The role of murein layer in the exclusion of toxic compounds from cell is improbable because of its structure and properties. Contrarily, G^- bacteria have a very thin murein layer that is linked from the outside part with the outer layer. The predominant component of this addition layer is lipopolysaccharide (LPS) composed of polysaccharide chains with six to seven saturated fatty acid bonds in glucosamine disaccharide structure. Thanks to these tightly packed saturated fatty acids, LPS has a very low permeability to hydrophobic compounds and thus can act as cell protection [51]. LPS chain plays a role in cell resistance as well. The study with *E. coli* mutants unable to synthesize these polysaccharides showed high sensitivity toward hydrophobic detergents [33]. Moreover, changes in LPS composition led to higher o-xylene resistance of *Pseudomonas putida*. LPS molecules with high molecular weight were replaced by a lower weight bands to adapt to *o*-xylene [36]. This concept of a protective function of LPS can be supported by a lower sensitivity of G^- bacteria toward various organic contaminants such as biphenyl, benzene, naphthalene, PCBs, and toluene [19, 29]. The amount and type of LPS molecules present in bacterial cell wall have crucial effects on the bacterial surface properties as hydrophobicity and adhesion with outer surfaces and substrates. The decrease of cell hydrophobicity generally leads to lower cell availability toward lipophilic contaminants and diminished permeability [61]. Some microorganisms that are capable of utilization of hydrophobic contaminants produce biosurfactants to increase bioavailability of such unique carbon sources [65]. Cell survival in adverse environment can be supported also by the addition of divalent ions (Mg^{2+} and Ca^{2+}). It is supposed that these divalent ions can diminish the charge

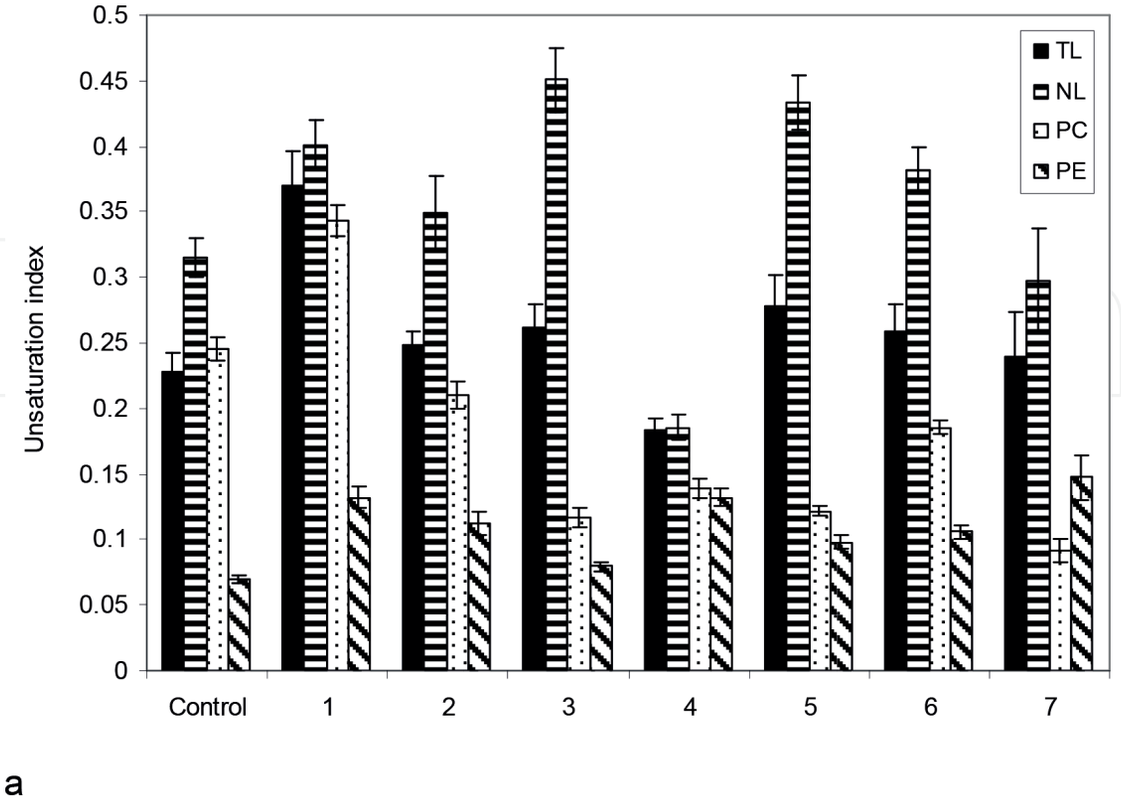
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repulsion of adjacent polyanionic LPS molecules with their electrostatic bond. Higher toluene resistance of *Pseudomonas* sp. was observed after the supplementation of cultivation media with divalent ions [33]. Toluene adaptation correlated with lowered surface hydrophobicity [51]. The removal of LPS molecules can lead to the loss of the resistance to toxic contaminants [66]. Although the penetration of external compounds is diminished by outer membrane, large number of small molecules can move through this compartment, thanks to protein canals. Changes in cell morphology in the presence of toxic compounds were observed in G^- [67] as well as in G^+ bacteria [68]. General responses of G^- bacteria to environmental stress were attributed to increase cell size. G^+ bacteria showed filamentous growth, increased cell volume, formation of endospores [63, 69], and production of unusual extracellular capsule [70].

2.6. The presence of terpene-containing plant matrices protected bacteria against the environmental stress and facilitated biodegradation of PCBs

Another efficient way how to cope with toxic compounds is to decrease their toxic effects with their biodegradation or biotransformation. The appropriate degradation enzymes, mono- or dioxygenase, are bonded to the inner part of bacterial cytoplasmic membrane. Bioaccumulation of hydrophobic compounds in cytoplasmic membrane is minimized with hydroxylation of these compounds. The usual degradation pathway begins with the incorporation of hydroxyl group into the pollutant structure [71–75]. However, increase in pollutant's polarity leads to its higher water solubility and higher availability to a microorganism itself. This situation usually leads to higher toxicity of the environment. Therefore, the microorganisms able only to modify toxic compounds probably cooperate with other organisms to achieve complete mineralization of contaminants into CO_2 and H_2O or at least transform the parent compounds into less or nontoxic intermediates [76].

Some compounds present in the nature can help bacteria to degrade the target pollutant [77–80]. The mechanisms of these compounds have not been described in detail yet. However, we observed that some of these compounds can diminish toxic effects of PCBs and their intermediates, namely chlorobenzoic acids, and consequently decrease bacterial adaptation mechanisms relating to membrane fatty acid composition. Then, bacteria were able to degrade PCBs nearly "without adaptation responses" which means that adaptation changes were observed only in a small extent because bacteria were "protected" in the presence of these compounds [81]. Plants rich in terpene contents belong to this group. Many studies including our research described the stimulation effects of ivy leaves, pine needles [82], eucalyptus leaves, tangerine, and orange peel [83–85] on biodegradation of hydrophobic pollutants. Potential use of natural plant matrices containing terpenes in the bioremediation of PCBs was studied in our previous works [15, 19, 81]. Our results clearly indicated the stimulation effects of terpene-containing matrices, namely ivy leaves and pine needles on bacterial growth in the presence of PCBs. The increase of fatty acids (FAs) content that is responsible for the increase of membrane fluidity was observed. Consequently, the smaller extent of necessary adaptation changes (*trans*/*cis* ratio of UFAs, *anteiso*/*iso* of branched FAs) was determined using addition of ivy leaves and pine needles into the defined mineral medium and the real polluted sediment, both contaminated with PCBs during degradation by bacterial isolate *Ps. stutzeri* (**Figure 5a**, **b**) and control strain *B. xenovorans*. More details can be seen in [11]. On the contrary, none stimulated



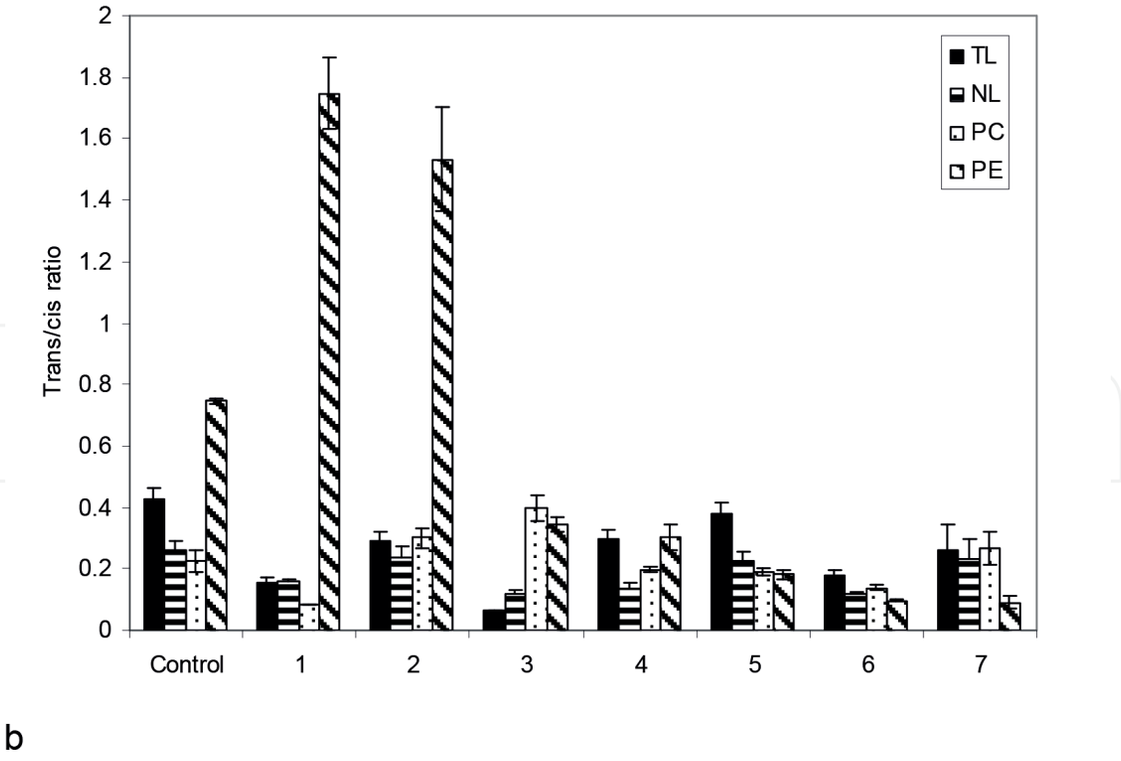


Figure 5. (a) The unsaturation index and (b) *trans*/*cis* ratio of fatty acids in total lipids (TL), nonpolar lipids (NL), and membrane lipid phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of bacterial strain *P. stutzeri*. Experimental sets: Cont, control experiment contains PCBs; 1, PCBs and biphenyl; 2, PCBs and carvone; 3, PCBs and limonene; 4, PCBs and ivy leaves; 5, PCBs and pine-needles; 6, PCBs and orange peel; and 7, PCBs and tangerine peel. Modified according to [81].

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and protected effects were observed in the presence of used synthetic terpenes, carvone and limonene, which corresponded with other papers [86–90].

It is important to note that the growth rate of anaerobic indigenous or incorporated bacteria is much slower when compared to that of the aerobic ones. Therefore, the adaptation mechanisms take more time and these bacteria are sensitive to organic compounds, e.g., solvents to a higher extent than aerobic bacteria [25].

2.7. Bioremediation of PCB-contaminated sediment using bioaugmentation (introduction of the adapted resistant bacteria) and biostimulation (addition of the natural plant terpenes)

Bacterial strains with pronounced degradation ability (that possess the *bph* gene encoding biphenyl dioxygenase starting the first step of PCB degradation—hydroxylation of PCB congeners) and ability to adapt and colonize in the adverse environment are the essential elements of successful bioremediation process. The use of the microorganisms to clean up polluted environments using their degradation ability is a rapidly expanding area of environmental biotechnology, namely bioremediation technologies. Bioremediation is an attractive, generally low-cost, innovative technology that represents a sustainable approach to removal of organic and inorganic pollutants. Bioremediation represents a perspective and prospective technique for decontamination treatment that involves application of microorganisms and/or plants for pollutant biodegradation or biotransformation. The two assisted bioremediation strategies—biostimulation and bioaugmentation are usually applied, when natural attenuation is not fast enough or complete enough (natural attenuation means the nonassisted reduction of contaminant concentrations in the environment through physical phenomena, chemical reaction, or biological processes). PCBs are generally subjected to both aerobic and anaerobic metabolism of bacteria. It is generally known that under aerobic conditions, biphenyl dioxygenase attacks biphenyl core and transforms PCB congeners into the respective chlorobenzoate and a pentanoic acid derivative. Under anaerobic conditions, PCB congeners are subjected to reductive dechlorination resulting in the intact biphenyl and some lower chlorinated PCB congeners. Both metabolic pathways are working only when the environmental conditions are optimal for the indigenous or introduced bacteria [91, 92].

Bioaugmentation can be defined as the technique for improvement of the metabolic capacity of the indigenous population to remove pollution by the inoculation, which means introduction of specific competent strains or consortia of microorganisms to the contaminated soil or sediments. Usually, the indigenous (autochthonous) or exogenous (allochthonous) bacteria are used. The basic premise for such intervention is to improve biodegradation of pollutants and save the time of treatment. Biostimulation involves addition of nutrients, trace minerals, electron acceptors, electron donors, or some inducers to improve the growth and then metabolic activity of the indigenous microbial population. Both approaches can be used under aerobic and anaerobic conditions, while the former is the prevailing case [93, 94].

Two strategies of assisted bioremediation, (a) bioaugmentation and (b) combined bioaugmentation and biostimulation, have been applied to degrade PCBs in the river sediment long-term exposed to PCB contamination sampled from the surroundings of a former PCB producer. A PCB-resistant bacterial strain *Ochrobactrum anthropi*, one of the two best

evaluated isolates related to adaptation mechanisms in the presence of PCBs with the minor adaptation responses (lower *trans*/*cis* ratio and higher *anteiso*/*iso* ratio) was used. One experimental set represented bioaugmentation treatment (introduction of bacteria 10 mg kg^{-1} of dry sediment into 5 g of contaminated river sediment flooded with 15 ml of defined mineral medium) and the second one combined bioaugmentation (introduction of bacteria 10 mg kg^{-1} of dry sediment) and biostimulation (addition of 15 g of terpenes containing ivy leaves cut into small pieces per kilogram of dry sediment) (for more details see [11]). Bacteria were introduced into the contaminated sediment to enhance the number of PCB degraders. The ivy leaves served as a stimulant agent of indigenous and introduced bacterial growth and as a protective agent against environmental stress caused by the presence of PCBs, as well as a potential PCB degradation inducer as structural analog of biphenyl (due to the toxicity not allowed to the environment as the inducer). Contaminated sediments treated with bioaugmentation and combined bioaugmentation and biostimulation were compared with the nontreated sediment (abiotic control experiment in which activity of the indigenous and introduced bacteria was inhibited by addition of 2.5% sodium azide). During the static 85 day-biodegradation at 28°C in the dark, the evaporated PCB congeners were captured on the sorbent SILIPOR C18 on the apparatus [95] and their amount was deducted from biodegraded one. The evaporation of PCB congeners was highest in the control experiment and lowest in the experiment with the addition of bacterial strain together with ivy leaves due to sorption. The results of specific PCB congener analyses revealed the degradation ability of adapted bacteria *O. anthropi* toward wide spectrum of chlorinated biphenyls. The initial amount of 12 determined PCB congeners was 40 mg kg^{-1} of dry sediment. Both lower (di-, tri-, and tetra-CBs) and higher chlorinated (penta-, hexa-, and hepta-CBs) congeners present in the industrial mixture of PCBs Delor103 were reduced during the bioremediation process. The higher PCB degradation was achieved during combined bioaugmentation and biostimulation (**Figure 6a**). Linearity for the in-time removal of PCB 101 (2,2′,4,5,5′-penta-CB) and PCB 118 (2,3′,4, 4′,5-penta-CB) by *O. anthropi* in bioaugmentation experiment was observed (not shown). The highest degradation in the experiment with ivy leaves was observed within the first 7 days when 5% (PCB 8) to 34% (PCB 180) degradation was achieved. The presence of ivy leaves in sediment led to higher biomass decrease within the first 7 days; however, after 42 days, the number of viable cells increased. Interestingly, the decrease of biomass within first 7 days of cultivation with ivy leaves was accompanied with the highest degradation rate. Ivy leaves could probably induce the activity of PCB degradation enzymes first and, after, the utilization of other carbon substrates present in sediment that they served as energy source. The degradation rate of PCBs removal accelerated after first 28 days in both bioaugmentation, and combined bioaugmentation + biostimulation experiments. The addition of ivy leaves stimulated PCB biodegradation which led to increased removal of PCB congeners (**Figure 6b**). The removal of overall PCBs was significantly higher when the combination of bioaugmentation and biostimulation strategy was used. Total degradation of PCB congeners in the sediment is presented in **Figure 6a**. Lower chlorinated congeners (PCB 4 and PCB 8) underwent transformation to a smaller extent. On the other hand, higher chlorinated congeners (PCB 118, PCB 138, and PCB 153) have been transformed to a higher extent. Low degradation of di-CB compared to the higher chlorinated congeners could be explained with the higher evaporation of di-CB compared to penta- and hexa-CBs that could

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diminish the amount of di-CB accessible to microorganisms. Addition of the ivy leaves increased mostly the degradation of PCB203, PCB8, PCB101, and PCB28 (18, 17, 15, and 14% increase compared to the PCB removal under the bioaugmentation conditions). At the end of bioremediation process, the highest degradation of PCB 18 (2,2′,5-tri-CB) in both remediation approaches was established (**Figure 6a**). Control experiment with suppressed bacterial

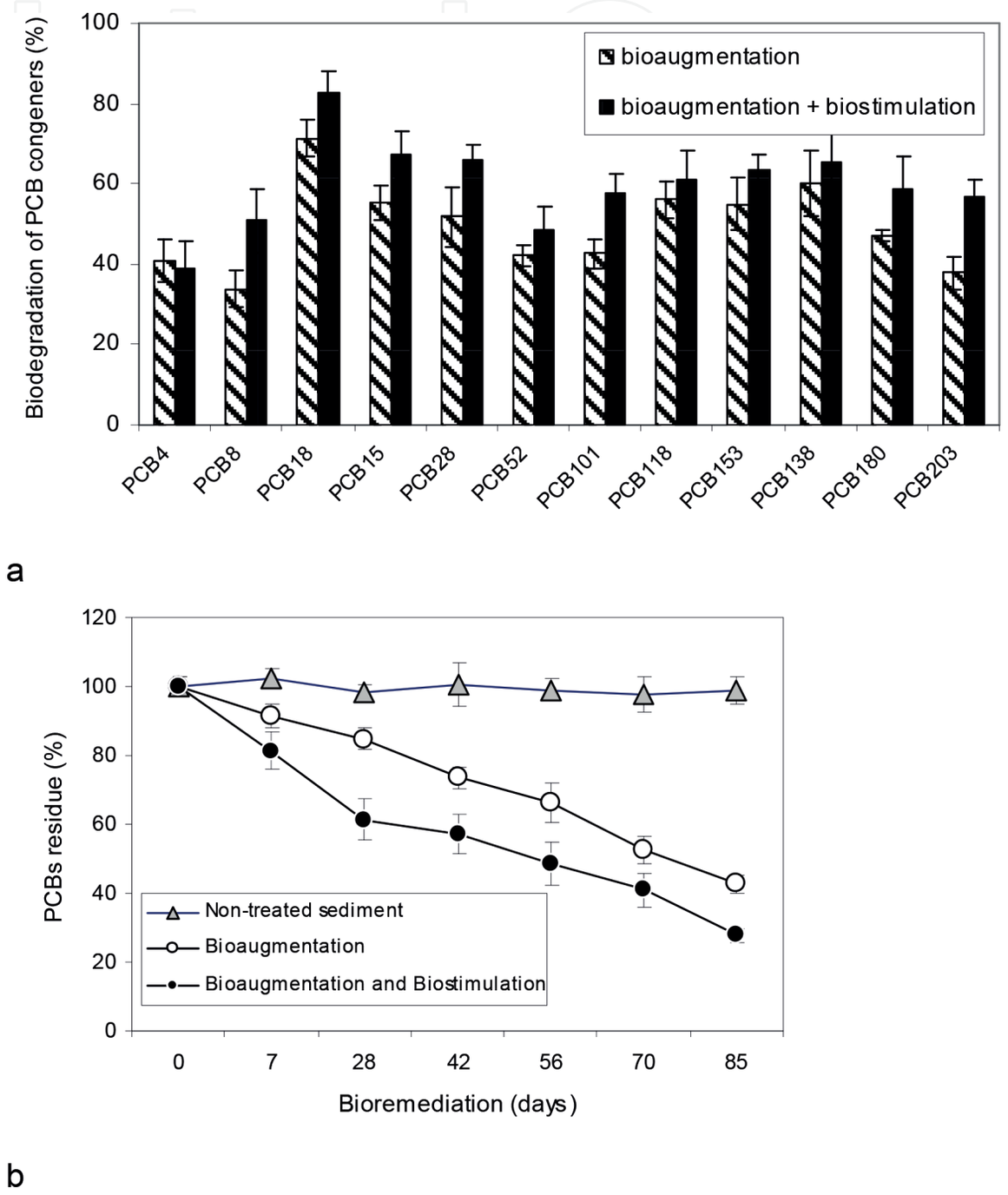


Figure 6. (a) Content of residual PCBs after 85-day bioremediation of PCB-contaminated sediment in the presence of introduced *Ochrobactrum anthropi*; (b) the change in the residual PCB content throughout bioremediation: bioaugmentation with *O. anthropi* and bioaugmentation + biostimulation with the addition of *O. anthropi* and ivy leaves. Control represented nontreated sediment with the inactive biomass. Modified according to [11].

growth revealed none or just very low PCB congener transformation caused probably by the abiotic factors (**Figure 6b**). **Figure 6b** shows also the overall change in the amount of PCB residues in sediment during the duration of remediation process. After 85 days, 27% of the initial PCB amount (40 mg of eight determined PCB congeners per kilogram of dry sediment) remained in sediment treated with a combination of bioaugmentation and biostimulation (with an addition of *O. anthropi* and ivy leaves). Sediment treated only with bioaugmentation (addition of *O. anthropi*) contained 1.5 times higher content of residual PCBs. The performed experiments confirmed the stimulatory effect of ivy leaves toward the bacterial growth and degradation ability of *O. anthropi* as well as on better adaptation to PCBs. The ability of *O. anthropi* to transform higher chlorinated PCB congeners in contaminated river sediment was established as well. These findings could be useful for bioremediation technologies in the decontamination of PCB polluted environment.

3. Conclusions

Many responses have been observed and confirmed in bacteria that counteract the effects of toxic environmental organic pollutants. Rigidification of the cell membrane is a consequence of cell adaptation mechanisms. The alterations in cytoplasmic membrane maintain ratio between bilayer and nonbilayer phospholipids (prevention against the environmentally induced formation of interdigitated structure) and keep the optimal phospholipids ordering to stabilize membrane fluidity. Another mechanism to protect bacterial cell is the efflux of toxic compounds from the membrane compartment. Toxic compounds affect not only cytoplasmic lipids but also cell proteins. This results in the development of special protein repair mechanisms by bacteria. Study of these adaptation mechanisms was the first step in selection of appropriate resistant bacterial strains, usually isolated from the contaminated area, and used for bioremediation application. Successful environment decontamination using biological approaches requires bacterial strains that can degrade particular (one or more) contaminants. Moreover, such strains have to be able to survive and adapt to adverse environment. Next step included the study of degradation potential of the most resistant strains. The resistant strain/consortium possessing appropriate degradation enzymes is the essential element of successful bioremediation. Both assisted bioremediation approaches, bioaugmentation and biostimulation, revealed to be perspective and prospective approaches of PCB decontamination. The degradation studies in artificial precisely defined matrices under the laboratory conditions (microcosms) could be applied in macrocosm and then after verification of strain/consortia degradation efficacy and survival ability and characterization of the optimal conditions for the successful decontamination process used in the field conditions.

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