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Occurrence of *ortho*- substituted PCBs in the environment and their biological activity

Bachelor thesis

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Annotation:

Ortho- substituted polychlorinated biphenyls (PCBs) are severe environmental pollutants with many effects on the living organisms. In this work, their fate, health effects on the organisms, biodegradation and proper handling in laboratory preparation of analysis is discussed. Their concentration in the soil was also measured.

Anotace:

Ortho- substituované bifenyly (PCBs) jsou závažné environmentální polutanty s mnoha vlivy na živé organismy. V této práci jsou rozebrány jejich vlivy na zdraví organismů, biodegradace a správné zacházení při přípravě analýzy v laboratoři. Byla změřena také jejich koncentrace v půdě.

Annotation:

Ortho-substituierte Polychlorierte Biphenyle (PCBs) sind schwerwiegende Umweltschadstoffe und beeinflussen auf viele Arten den lebenden Organismus. In dieser Arbeit werden ihr Verlauf, gesundheitliche Auswirkungen auf den Organismus, ihre biologische Zersetzung und der richtige Umgang bei Versuchsvorbereitungen im Labor diskutiert. Weiters wurde auch ihre Konzentration in der Erdboden gemessen.

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České Budějovice, 20. 12. 2010 .....

Lucie Ouředníková

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Finally my thanks go to Mgr. Jiří Novák, Ph.D. from Recetox Brno who showed me the real research “in practice”, and also to my family and friends for their support.

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A = Aroclor (trade name for technical mixture of polychlorinated biphenyls)

AHH = Aryl hydrocarbon hydroxylase

BBTT = Bacterial bioluminescence tests of toxicity

BPDM = Benzphetamine-N-demethylase

BSAF = Biota- sediment accumulation factor

DA = Dopamine

EDCs = Endocrine disrupting chemicals

EROD = ethoxyresorufin-o-deethylase

GJIC = Gap Junctional Intercellular Communication

GPC = Gel Permeation Chromatography

IC_{50} = Concentration causing 50% inhibition

ISO = International Organization for Standardization

PBS = Phosphate buffer saline

PCB = Polychlorinated biphenyls

RyRs = Ryanodine receptors

T_4 = Thyroxin

TCCD = 2,3,7,8- tetrachloro dibenzo- *p*- dioxin

TSB = Trypton soya broth

TSH = Thyroid stimulating hormone

TTR = Transthyretin

1. Introduction

Polychlorinated biphenyls (PCBs) are a class of biologically active industrially produced chemical compounds. Nowadays they are known as global environmental contaminants which are persistent and their ubiquitous nature has prompted many studies of their potential health hazards (Smithwick, et al., 2003).

PCB production on a small scale began in 1881 in the United States, commercial mass production in America was initiated in 1929 under the trade name Aroclor®, other trade names are Clophen (Germany), Kanechlor (Japan), Phenoclor and Pyralene (France). In Czechoslovakia PCBs were produced mainly in n.p. Chemko Strážské and Spolana Neratovice under trade names of Delor, Hydelor and Delotherm. Major trade names are summarized in Table 1 (Urbaniak, 2007). With the respect to very large usage of PCBs and to the production area, which was in the Eastern Slovakia, the Czech Republic and Slovakia belong among countries with the highest handicap of PCBs in Europe. In Czechoslovakia approximatelly 6200 tons of PCB were produced during 1959- 1984.

Table 1: Major trade names of PCBs

Apirolio	Chlorextol	Elemex	Montar	Saf-T-Khul
Areclor	Chlorinol	Euracel	Nepolin	Santotherm
Aroclor	Chlorphen	Fenchlor	No-Flamol	Santovac
Arubren	Clophen	Hivar	Pydraul	Siclonyl
Asbetol	Delor	Hydol	Pyralene	Solvol
Askarel	Diaclor	Inclor	Pyranol	Sovol
Bakola	Duconol	Iterteen	Pyroclor	Therminol
Biclor	Dykanol	Kennechlor	Pheneclor	

PCBs were valued for their heat stability and thus widely used industrially as flame-retardants and dielectric fluids in transformers and capacitors (Erickson, 1997). They were soon also employed in such diverse applications as plastics, nautical paints, microscope oil, pesticides, copy paper, adhesives, and sealants. Unfortunately, the same chemical properties

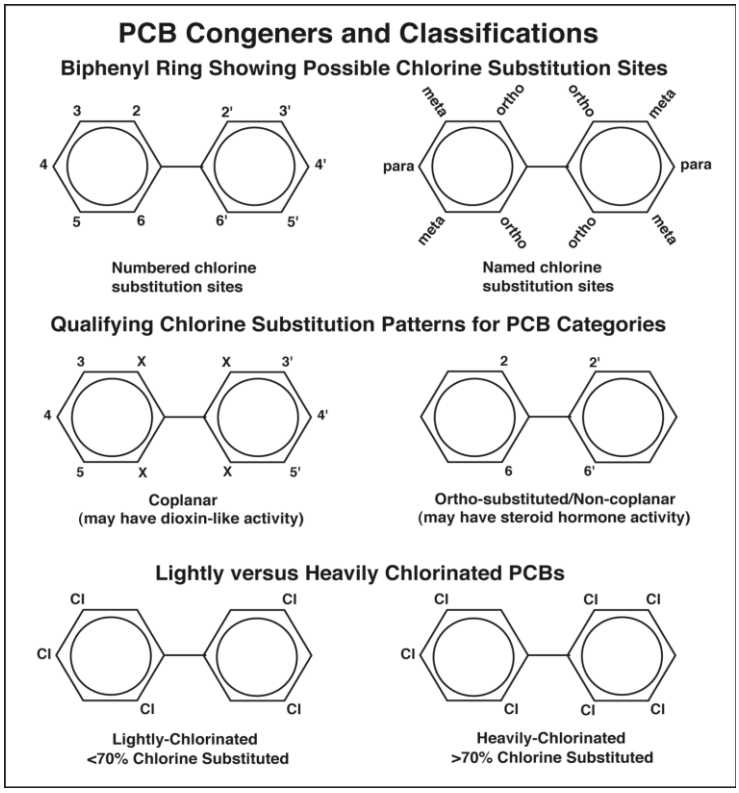
that made PCBs valuable chemicals for industry also contribute to their toxicity.

Production of PCBs was banned in 1977 in the USA, in Czechoslovakia in 1984, but in spite of this fact the bioaccumulation, continued dumping, and landfill leakage create a pervasive toxic environment in both urban and rural landscapes across the world. Biotransfer via migratory organisms and oceanic currents has resulted in the paradoxical findings that pristine regions far from primary contamination can contain dangerously high levels of PCBs (Steinberg, 2007).

PCBs congeners have various biological activities influencing very wide scale of processes in the organism, e.g. reproduction, immune system, metabolism, hormone production. PCBs are able to bio-concentrate in human blood, adipose tissue and breast milk (Mullin, et al., 1981). Some of them are also very potent inducers of tumors and carcinogenesis in liver (Kannan, 2000) and other organs.

The structure of PCBs is based on a biphenyl ring, i.e. a molecule composed of two benzene rings to which 1 up to 10 chlorine atoms are attached to. For better illustration see Figure 1.

Figure 1: Structure of congeners and their classification



There are 209 possible PCB permutations (congeners) of chlorine substitution, defined by the number and positions of chlorines on the biphenyl frame. In order to simplify the nomenclature of PCBs, systematic numbering has been introduced by Ballschmiter and accepted by IUPAC. Each of the congeners has been designated with a number from 1 to 209 (Ballschmiter, et al., 1980). For the full list of congeners see Attachment 1.

The PCB molecules are mostly non-planar and their physical properties are very important for their characteristics, like for example biological activity or the way of possible degradation. The major physical properties are determined by: the degree of chlorination, the three-dimensional structure of the molecule and the substitution pattern of chlorine atoms on the biphenyl ring. The substitution pattern, therefore is another wayto distinguish among the congeners - more specifically, if there is substitution on *ortho*- position or not. The reason for this is that the properties are largely dependent on the number of *ortho*-substituents. According to this, PCBs can be separated to groups following the number of substitutions, e.g. Non- *ortho*, Mono- *ortho*, Di- *ortho,* Tri- *ortho* chlorinated biphenyls etc.

This thesis is mainly set to PCBs which are chlorinated in the *ortho*- position. It was believed for a long time that these compounds are not as dangerous as the other congeners but recently it was found out that these compounds are as dangerous as other polychlorinated biphenyls or even more. They very easily bio-accumulate in nature, they have antiestrogenic effects (Oh, et al., 2007) and they are also carcinogenic.

2. Occurrence and fate of ortho- substituted PCBs in the environment

In the 1970- 1980s, the production of polychlorinated biphenyls underwent very strict control and in many countries their production was banned. However, a release of PCBs from contaminated reservoirs still exists. Due to their lipophilic character they remain in soil, water and sediments for many years. Mackay, et al., in 1992 published an estimation for how long selected PCBs stay in the environment. For illustration see Table 2. Moreover, they can bioaccumulate in cells and pass up through a food chain.

For concentration of PCBs in the environment see Table 3 (WHO/EURO, 1987).

Table 2: Half- time of remaining of selected PCBs

Degree of chlorination	Air	Water	Soil	Sediment
1	1 week	8 months	2 years	2 years
2	1 week	8 months	6 years	2 years
3	3 weeks	2 years	6 years	6 years
4	2 months	6 years	6 years	6 years
5	2 months	6 years	6 years	6 years
6	8 months	6 years	6 years	6 years
7	8 months	6 years	6 years	6 years
8	2 years	6 years	6 years	6 years
9	2 years	6 years	6 years	6 years
10	6 years	6 years	6 years	6 years

Table 3: Concentration of PCBs in the environment

Environ. Compartment	Concentration	Environ. compartment	Concentration
Atmospheric air	0,1-20 ng/m^3	**Plankton**	10-20 000 ng/g
Water	0,001-908 ng/l	**Invertebrates**	10-10 000 ng/g
Sediments	1,1-6000 ng/gsm	**Fish**	10-25 000 ng/g
Bird eggs	100- 500 000 ng/g	**Humans**	100-10 000 ng/g

2.1. Occurrence in the air

The atmosphere contains a low percentage of PCBs, however, it is believed that it serves as the most common pathway of PCB transportation to terrestrial and aquatic ecosystems (Lee, et al., 1996). PCBs can in this way undergo a long-range atmospheric transport, moving from source to different locations where they have not been produced or used. Therefore, PCBs were for example found in Arctic char (Braune, et al., 2005) or in the Antarctic (Weber, et al., 2003).

In 1989 Dunkier and Bouchertall showed in their study that lower chlorinated PCBs, as mono- and di- chlorobiphenyls, are dominant in the filtered air. It is due to their low solubility, they are not washed out from atmosphere by rain water. On the other hand, higher chlorinated biphenyls can be adsorbed by rain drops and therefore removed from atmosphere.

2.2. Occurrence in soil, sediments and water

Except highly industrialized areas, the contribution in soil to total amount of PCBs is small. However, landfills and other PCB- contained soils are large PCB reservoirs from which the PCBs can be spread out to other areas or groundwater.

The contamination of water can be done through sediment, i.e. desorption, erosion and gas convection, or through air- water exchange from atmosphere. Lower chlorinated PCBs are sorbed less strongly than the higher ones, therefore they tend to leach more in soil.

PCBs found in sediments are predominantly the higher chlorinated ones, because they are less soluble and they posses stronger adsorption on soil particles. These highly chlorinated PCBs can be passed into the food chain. This process can be described by the biota-sediment accumulation factor (BSAF). The highest BSAF value has the mono- *ortho*-substituted congener 118, the lowest PCB 156. On the other hand, sedimentation can greatly reduce the concentration in water by binding PCBs congeners to particulates (Urbaniak, 2007).

2.3. Occurrence in animals and in human body

PCBs are present in air, soils and sediments, thus it is obvious that there is a great possibility of releasing previously deposited PCBs to animals. Primary environmental exposure to PCBs can be done through the contaminated food sources, but also through an accidental exposure like inhalation or skin contact. It can lead to chloracne, skin rashes, respiratory problems, irritability, fatigue, nervousness, and insomnia.

Next to the abnormal chemical stability, another important property of PCBs is their lipophilic nature. It enables them to accumulate in fats and other tissues because many organisms lack an adequate catabolic defense against this man-made chemical (Steinberg, 2007). This property facilitates the bioaccumulation of PCBs in organisms through the food chain (Kidd, et al., 1998), tissue sequestration but moreover, these compounds can be passed from mother to offspring in uterine or oviductal fluids, or in lactate, i.e. accumulate in animal breast milk and cross the placenta. This can result for example in developmental delays and cognitive impairments in offspring of PCB exposed individuals (Patadin, et al., 1999; Jacobsen, et al., 1996).

2.3.1. Influence of PCBs structure on biological activity

As mentioned in the Introduction, the most important characteristics influencing the PCBs properties are the substitution pattern, degree of chlorination and the three-dimensional structure of the molecule.

PCBs as many other compounds are toxic. The magnitude of the toxicity can vary by order of magnitudes. Therefore a toxic equivalent factor (TEF) was introduced. As the reference compound, the most toxic compound known was used, namely 2,3,7,8-tetrachloro dibenzodioxin (TCDD). With this, TEF, the toxicity of compounds, can be expressed in a single number.

The classic view was that the more toxic PCBs were those with no (the so- called coplanar PCBs) or few ortho chlorines, and included those that were agonists of the AH receptor (Safe, 1990). The first obvious exception to that concept was observation that PCBs with multiple *ortho* (and *para*) chlorines were active as promoters of hepatocarcinogenesis

(Glauert, et al., 2001). They have neurotoxic effects, anti (estrogenic) effects (Gierthy, et al., 2001), calcium signaling effects (Pessah, et al., 2001) and etc.

2.3.1.1. Influence of a degree of chlorination

As an example of influence of degree of chlorination on PCBs properties, it was found out that highly chlorinated PCBs containing at least 7 chlorine substitutions have a longer half- life within living organisms as well as in the environment (Platonov, et al., 1975). On the other hand, lower chlorinated PCB homologues are readily metabolized *in vivo,* and thus do not bio-concentrate and have a shorter half- life. These PCBs easily volatize and may be inhaled in aerosol form (Hermanson, et al., 1989), or ingested after being phytoconcentrated in agricultural plant stock (Thomas, et al., 1998).

2.3.1.2. Influence of a substitution pattern

Substitution pattern can be also one of the types how to divide PCBs- if the compound has a substitution in *ortho* position or not. According to this, the PCBs can be separated into groups according to their number of *ortho* positions, e.g. non- *ortho*, mono- *ortho*, di- *ortho* etc. The reason for this is that the properties are largely dependent on the number of *ortho*- substituents.

2.3.1.2.1. Non- ortho and mono- ortho substituted PCBs

Non- and Mono- *ortho* chlorinated biphenyls have similar properties to 2, 3, 7, 8- tetrachloro dibenzo- p- dioxin (TCDD), resemble similar toxic responses in their biological action. In general, they are said to be the most toxic PCBs (Ahlborg, et al., 1994). They revealed the following syndromes: decreased reproductive efficiency, changes in liver morphology, changes in plasma lipid concentration, hepatic porphyry, decreased immune competence, dermatological effects and production of tumors in liver (Kannan, 2000). They also affect numerous enzymes by induction, including hepatic and extra hepatic drug metabolizing enzymes. They are the most potent inducers of Cytochrome P-450IA1 and Cytochrome P-450IA2 hem proteins, ethoxyresorufin-O- deethylase (EROD) activity and Aryl

hydrocarbon hydroxylase (AHH). These compounds are the most potent inducers as well as the most toxic (Kannan, 2000). For description of the influence on AHH see Part 2.3.

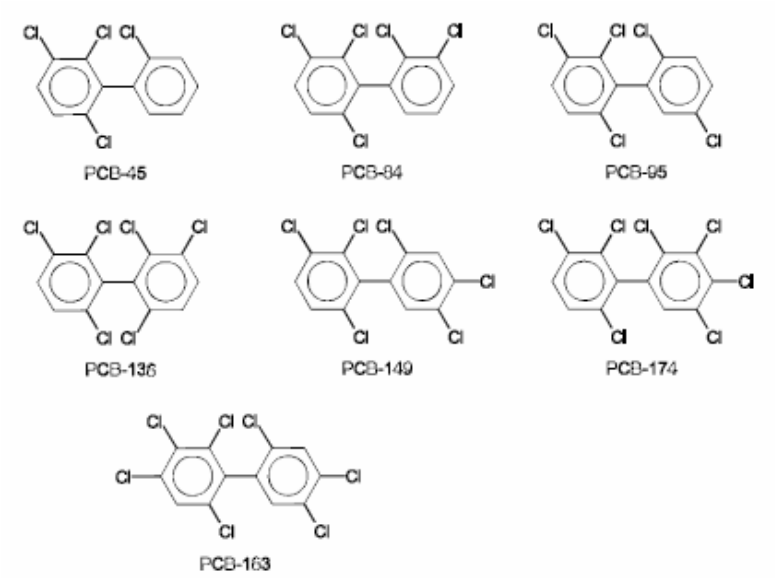
2.3.1.2.2. Di- ortho substituted PCBs

Among di- *ortho* PCBs belong e.g. PCB 168, 170, 180, 190, 191, 194 and 205. Because of the steric interactions of Di- *ortho*- chloro substituent, the biphenyl ring coplanarity is markedly inhibited and presumably decrease binding to the cytosolic receptor protein. Therefore these compounds are not inducers of AHH (or cytochromes P-450IA1 and P-450IA2) or elicit typical responses of TCDD (Safe, et al., 1985). The toxicity has not been systematically investigated, but two compound members of this group can be porphyrinogenic after long term feeding studies, and i.e. PCB 128 and PCB 137. The last mentioned one is a major component of commercial PCBs and preferentially bio concentrate in human blood, adipose tissue and breast milk (Mullin, et al., 1981).

2.3.1.2.3. Tri- ortho and tetra- ortho substituted PCBs

Tri- *ortho*- and tetra- *ortho*- PCBs may exist as stable atropisomers due to limited rotation about single bonds and the steric strain barrier is high enough that the conformers can be isolated from each other (Kaiser, 1974). There is a great increase in the energy barrier to racemization, i.e. quite high rotational free energy barrier – for tri- *ortho* ΔG= 176.6 – 184.8 kJ/mol and for tetra- *ortho* ΔG was about 246 kJ/mol. This made the atropisomers stable to racemization under physiological conditions but also under high temperatures (Robertson, et al., 2001). Kaiser (1974) predicted that 19 PCBs will exist as stable atropisomers at room temperature. The number of PCB congeners raises up to 228. Seven of them are “environmentally important” because they occur in Arochlor and in environmental samples (Hansen, 1999) but the toxic effects are unknown. These congeners are: PCB 45, 64, 95, 132, 149, 174, 183. Structures of some of them are showed in Figure 5. As PCBs mentioned before, tri- and tetra- *ortho* PCBs they also induce a Cytochrome P-450, influence ethoxyresorufin- O- dethylase (EROD) activity and benzphetamine- N- demethylase (BPDM) activity. Until recently, the toxicologists have paid a very little attention to PCBs with multiple *ortho* chlorine atoms.

Figure 2: PCB congeners forming stable atropisomers



2.3.2. Occurrence in animals

Many studies on the bioaccumulation of *ortho* PCBs in organisms proving that PCBs are present all over the world have been done. Especially aquatic invertebrates assume an important role in the cycling of PCBs within and between ecosystems. They consume phytoplankton, zooplankton and other biota in which PCBs from water and sediment tend to bioaccumulate.

Surprisingly unexpected large differences in the bioaccumulation of closely related PCBs have been documented. Occurrence of *ortho* PCBs is dependent on the lipophicility of the compound which is measured as log K_{OW} value, where K_{OW} is 1- octanol/ water partition coefficient. And of course, it is also dependent on the steric effects resulting from different substitution patterns of chlorines (Shaw, et al., 1984).

The bioaccumulation potential of three highly toxic coplanar PCBs (PCB 77, PCB 126, PCB 169) was investigated using green- lipped mussels (*Perna viridis* Linneaeus) as a biondicator at two locations in Hong Kong (Kannan, et al., 1989). Kannan et al. (1989) found out that non-ortho chlorine substituted coplanar PCB congeners had a slow uptake and clearance and on the other hand coplanar PCBs are highly bioaccumulative in lower

organisms. And because the mussels are not expected to be somehow unusual in bioaccumulation of PCBs, it appears most likely that these highly toxic and persistent PCBs congeners are present in all aquatic organisms, and may reach higher consumers (including humans) with the respect to toxicological concern (Kannan, et al., 1989).

Organochlorine pollution was also studied in fish from different parts of the river Seine, upstream and downstream from Paris (Chevreuil, et al., 1995). They tested two different species living around nine stations: roach (*Rutilus rutilus)* and perch (*Perca fluviatilis)* considering the relationship between fish, water and sediments and also influence of persistent compounds on the composition of the fish population. As a result it was found out that concentration of PCBs in downstream fish is much higher (~ 14 000 μg/kg) than in the upstream area (~3000 μg/kg) and that the downstream roach showed the most significant lipid content. It is in accordance with present study done by Boughida (1992) who described a faster growth of roach around this area. Except this, the PCB concentration expressed per unit of lipid weight proved that in this area the contamination is higher. And moreover there is really a correlation of a content of lipophilic PCBs to the lipid levels (Devaux, et al., 1987).

In the study, the content of pesticides and metallic micropollutans were also measured. It was found out that PCBs are the main pollutants, especially PCB 153 and PCB 138. They constitute 20% and 11% of all 15 components tested. Again, this was in accordance with the previous study done by Barcelo et al. (1989) that PCB 153 makes 22% of the total PCBs found in fish.

Many other not only of *ortho*- substituted PCBs in fish, e.g. in Baltic sea, in Netherlands, in the USA (in Saginaw River, Michigan) and their bioaccumulation and other effects on the living organism.

As another prove of presence of PCBs in the environment, respectively in hydrosphere, found in last decades, the mass mortalities in aquatic mammals were reported.. -the North Sea harbor seal epizootic of 1988, the US bottle nose dolphin of 1987-1988, Baikal seals in the same period of time and Mediterranean striped dolphin in the period of 1990-1991 (Kannan, 2000). As it was mentioned at the beginning of this project, the PCBs can cause an immunosuppression. And this was with a very high probability the case here- the mammals were infected by a virus due to the immune suppression induced by toxic PCBs.

2.3.3. Occurrence in human body

PCBs can occur in the human body from various sources. It is said that people receive 90- 99 % of PCBs from their food, mostly from fish, beef and milk.

However, PCBs are also introduced due to an accidental exposure as skin contact or inhalation, as well as transgenetical transfer from mother to the offspring. Because all the compounds due to their biological and chemical properties are persistent, they accumulate in the human body in all lipid tissues.

Body is effected in many ways- PCBs are neurotoxic, cause hormone disruption or can influence the behavior. It is also said that these compounds are carcinogens and possible mutagens. They cause hepatocelular carcinoma of the liver, porphyry and immunosupression. Its important property is an ability of activation of liver microsomal enzymes catalyzing a metabolic degradation of a large scale of exogenic and also endogenic substrates.

2.3.3.1. Biological activity

Main and well studied biological activity of PCBs is through induction of family of enzymes of cytochrome P-450, enzymes called isozymes.

Cytochrome P-450 enzymes are present in the inner membrane of mitochondria and also in endoplasmatic reticula and their main function is to metabolize and eliminate the xenobiotics from the organism. As was mentioned, these enzymes are activated through induction, i.e. the production by cells can be increased by various inducers, mainly by xenobiotics. That results in increasing activity of enzymatic complexes in which the activated cytochrome is involved. In the case of PCB, the most interesting is a complex of aryl hydrocarbon hydroxylase (AHH).

The problem with cytochrome P-450 is that its function is dependent on the surrounding factors, as a nature of foreign compound, the route into the organism, specific tissue susceptibility, the isozymal spectrum of induced P-450, and the ratio between P-450 and other related enzymes and compounds (Okey, 1990; Farrell, et al., 1990). It is also dependent on the structure of the compound. Therefore the P-450 may inactivate and

eliminate toxic xenobiotics but on the other hand it can also convert the foreign compound to more toxic or carcinogenic one. And as a result, the P-450 may prevent intoxication or cause intoxication, and may protect against chemical carcinogenesis or increase the risk of cancer (Lang, 1992).

After finding out of relationship between the structure, AHH induction activity and toxicity, PCBs were classified into groups.

Co- planar PCBs, i.e. they don't have any *ortho*- substitution, are in general the most potent inducers of cytochrome P-450 and thus also of AHH (and EROD) because of their similarity to 2,3,7,8- TCDD.

Mono-*ortho* PCBs, with one *ortho*- chlorine (congeners 105, 114, 118, 123, 156, 157, 167 and 189) are also potent inducers of AHH activity, they are mixed-type inducers and in many of them toxic effects similar to those of 2,3,7,8,-TCDD have been proved (Lang, 1992).

Di- ortho PCBs include congeners 128, 137, 138, 153, 158, 166, 168, 170, 180, 190, 191, 194 and 205. They are not very potent inducers (Sawyer, et al., 1982).

PCBs with more *ortho*- substitutions can also to some extent induce in high doses (10- 100 mg/kg) AHH and EROD activities *in vivo*, but their inducing potencies are considerably lower in comparison with AHH-type inducers between PCBs or 2,3,7,8-TCCD.

The biological activity is measured as a biological potency. It is a dose or concentration that induces a half of the maximum value, it can be ED_{50} (= effective dose) or EC_{50} (= effective concentration). Owing to the similarity of toxic responses and mechanisms of the "toxic" PCB congeners and 2,3,7,8-TCCD, the biological potency is often expressed relative to that of TCDD. Such potency is described as a toxic equivalence factor (TEF). This TEF says how much TDCC would have the same effect as used or contained PCB. If these values are compared, it is said there are 12 toxic congeners from all 209. It does not mean that the others are harmless but it shows there are great differences in toxic potencies between them.

2.3.3.2. Effects in the human body

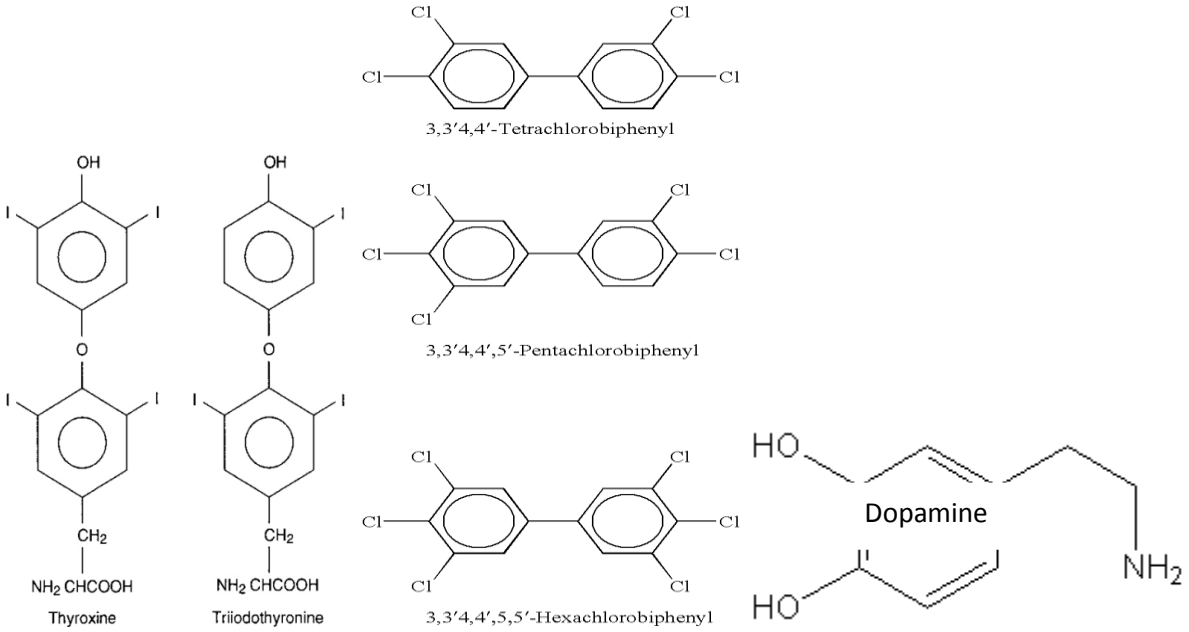
There are many proves that PCBs, including *ortho*, are very dangerous and

they have great effect on the human body. For example after two accidents of PCB poisoning caused by ingestion of edible oil in Japan and in Taiwan the symptoms of poisoned people were: liver damage, neurological symptoms, dermal lesions, damage to the endocrine system, immunodeficiency and reproduction disorders (Lang, 1992).

Moreover, many studies were done on this topic and they proved and found other effects. For example it was found that *ortho*- substituted congeners alter dopamine metabolism (Shain, et al., 1991), stimulate insulin release from RIMm5F cells (Fischer, et al., 1999), kill cerebellar granule cell neurons (Carpenter, et al., 1997) and so on.

In the following part the most important effects of PCBs on human body are discussed.

Figure 2: Similarity of some important hormones to PCBs- in here for better illustration example of ortho substituted PCBs and a hydroxylated PCB (sources: http://content.answcdn.com/main/content/img/oxford/Oxford_Sports/0199210896.thyroid-hormone.1.jpg; http://upload.wikimedia.org/wikipedia/commons/b/b0/Dopamine.png; www.freepatentsonline.com/6544773-0-large.jpg)



2.3.3.2.1. Endocrine disruptions

The most documented pathway is through the endocrine disruption. PCBs belong to the class of endocrine disrupting chemicals (ECDs). EDCs are natural or man-made chemical compounds that when introduced to a natural biological system, disrupt endocrine signaling at the synthesis, transport, signal transduction or catabolism stages of hormone activity (Steinberg, 2007). Lower chlorinated Aroclor formulations and *ortho*-substituted PCB congeners are commonly found to be estrogenic (Bitman, et al., 1970).

An important aspect in inducing estrogenic response is a number and ring position of the *ortho*-chlorine substitutions (Arcaro, et al., 1999). Substituted congeners with at least 1 chlorine on each ring are estrogenic. For example congener which has both ortho substitutions on the same ring, shows no activity, suggesting a requirement for *ortho* substitution on each of the biphenyl rings and the resulting non coplanar configuration. And higher chlorinated dioxin-like PCB tend to be anti- estrogenic, however these distinctions are not absolute (Steinberg, 2007).

In general it is very difficult to monitor direct effects of PCBs on endocrine system because it can be affected in various ways, starting from receptor levels, metabolic steps to concrete steps of feedback regulation. On the other hand many affects have been proved, including the estrogen and androgen system (Golden, et al., 1998), the thyroid hormone system (Ingbar, 1985; Brouwer, et al.; 1998), retinoid system, corticosteroid system and so on. In addition, as was mentioned before, non- *ortho* and mono- *ortho* PCBs exerts their effects mainly through the aryl hydrocarbon receptor (AhR) pathway.

To illustrate how the effects of PCBs are somewhat complex and they have many possibilities how to affect the functioning of the body, the next part shows the PCBs affecting one of the most important hormones, i.e. the thyroid hormone.

2.3.3.2.1.1. Thyroid hormones disruptions

Thyroid hormones are essential for normal body metabolism, growth, and development including reproduction, maturation, and aging; therefore, fluctuation of thyroid homeostasis are especially are especially important in developing animals during the critical phases of growth (Ingbar, 1985).

PCBs are structurally similar to the biphenyl structure of thyroid hormones. PCBs interfere with all levels of thyroid signalling, metabolism (Bastomsky, et al., 1976), and thyroid physiology (Langer, et al., 2003; Kilic, et al., 2005), as well as circulating thyroid hormone levels (Gauger, et al., 2004).

Acute exposures to these PCBs cause severe reductions in serum total T_4, thyroxin, and hypothalamic dopamine (DA) concentration without causing changes in serum thyroid stimulating hormone (TSH) levels (Desaulniers, et al., 1999). There are several mechanisms by which by which *ortho*- PCBs may reduce circulating T_4 levels including binding to a T_4 transport protein, transthyretin (TTR) (Chauhan, et al., 2000), increasing tissue uptake of T_4 (Capen, et al., 1991), and possible mechanism related to interactions with thyroid hormone receptors (McKinney, et al., 1994). There is also perturbation intracellular Ca^{2+} homeostasis by targeting ryanodine receptors (RyRs) (Pessah, et al., 1999). This suggests that some PCBs may disrupt T_4 through mechanisms related to intracellular calcium flux. However, the exact mechanism of action of these PCBs is not clear. Possible relevant targets can be hypothalamus because of known effects of these PCBs on DA content (Khan, et al., 2002), as well as anterior pituitary gland because of known effects of more labile PCBs (Jansen, et al., 1993) and the partial dependence on RyR- mediated calcium regulation (Ingbar, 1985).

Because thyroid hormone signalling is important for normal brain development, PCB depletion of circulating thyroid hormone may cause permanent brain abnormalities (Kimura-Kuroda, et al., 2005). It was found out that hypothyroidism during fetal and early neonatal life may have profound adverse effects on the developing brain (Crisp, et al., 1998). This may result in reduced axonal and dendritic sized and complexity (Stein, et al., 1991). It is well known that perinatal exposure of rodents to PCBs mixtures causes changes in dopaminergic and serotonergic neurotransmitter concentration and metabolism (Seegal, et al., 1992). For example, in a perinatal exposure study (Lilienthal, et al., 1997), similar influences were observed on the serotonergic system by PCB 77 and thyroid suppressive agent 6-n-propyl-2- thiouracil. Retarded behavioral and cognitive development and profound deafness are consequences of fetal hypothyroidism (Freeman, et al., 1995).

In addition to thyroid hormone, also levels of serum prolactin can be depressed. Coburn et al (2005) found a link between PCB exposure and altered vasopressin

release (Coburn, et al., 2005). As well as uterine oxytocin production was impaired by PCB exposure in a non-estrogen dependent mechanism (Mlynarczuk, 2005).

2.3.3.3. Other effects of PCBs

As it was mentioned, the PCBs have very large effects on the body. They influence the proper functions of receptors, organs, hormones. Therefore they can influence the behavioral, reproduction and moreover they can induce tumors and cancer.
One more aspect which is also very important and interesting could be a monitoring of influence of PCBs on intracellular communication.

2.3.3.3.1. Intracellular communication influenced by PCBs

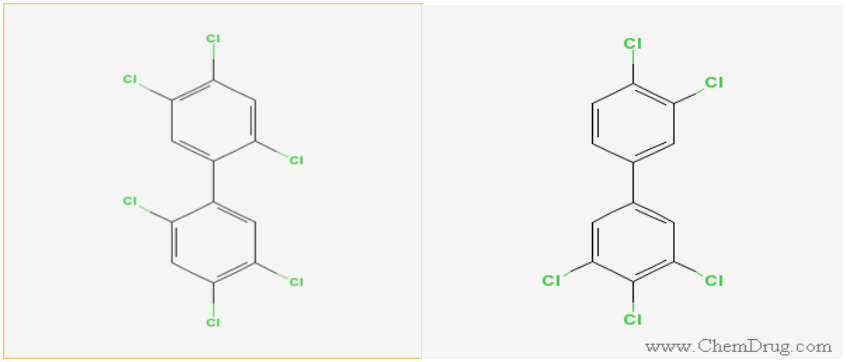
Intracellular communication, i.e. communication between cells, is dependent on permeable intracellular junctions, known also as gap junctions. These junctions facilitate the transfer of ions, metabolites, nucleotides but also signals between cells. Inhibition of this gap junction intracellular communication (GJIC) belongs among critical epigenetic events of tumor promotion. It was found out that di-*ortho*- substituted PCBs are quite potent inhibitors. They do not significantly activate AhR because of their non- coplanar structure but they exhibit a different spectrum of toxic modes of action, such as modulation of steroid hormone and Ca^{2+} - induced intracellular signaling and etc. They also have been linked to tumor promotion (Brouwer, et al., 1999; Dean, et al., 2002).

There are several steps which are very important to promotion of cancer. One of them is the removal of an initiated cell from the suppression of growth by neighboring cells through the intracellular transfer of signal transductions via gap junction. A critical step in this part is the down regulation of gap junction intracellular communication (GJIC) (Ruch, et al., 2001). Therefore the inhibition of GJIC can be assumed to be a representative marker of tumor-promoting potency for given compound (Rosenkratz, et al., 2000). The mechanism is not known.

The inhibition of GJIP by non- coplanar PCBs has been studied in Brno, in Recetox, Masaryk University by Miroslav Machala, Ludek Blaha and Jan Vondracek. In their study in 2003, they tried to determine relative potencies of a series of environmentally

relevant PCB congeners to inhibit GJIC and they also compared the effects of some coplanar PCBs against non- coplanar, e.g. PCB 153 and PCB 126.

Figure 4: PCB 153 and PCB 126 (source www.ChemDrug.com)



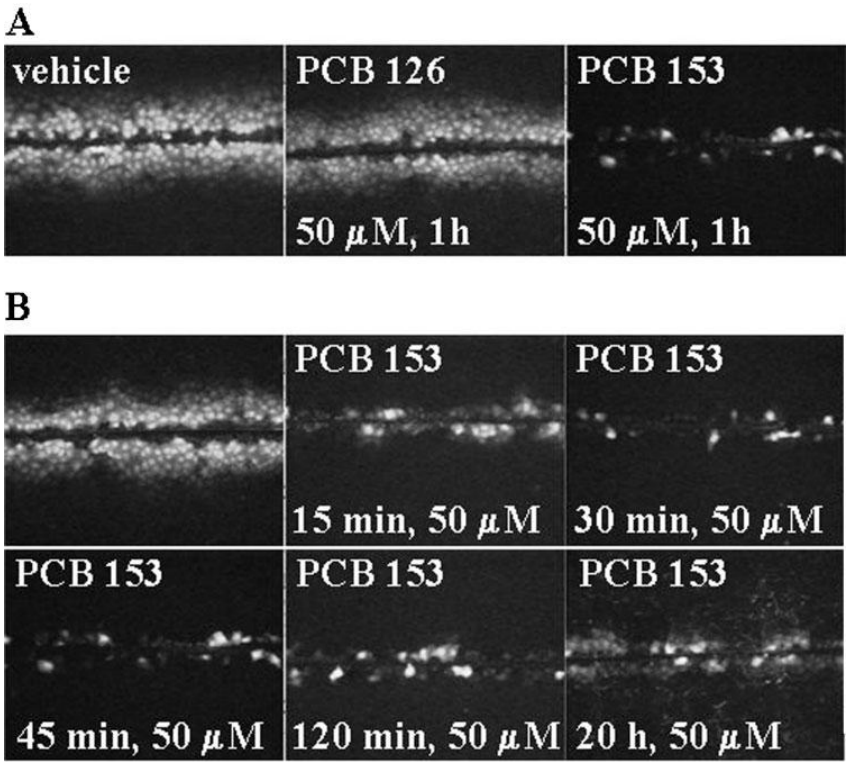
As a result, it was proved that there is a great influence of structure alternations – the non-coplanar PCBs with three up to six chlorine substitutions at *ortho* position are the most potent inhibitors of GJIP. Non- *ortho* PCBs have no effect but they alter the interaction with AhR as was mentioned above (see Part 2.3.1.2.1.).

In the comparison of PCB 153 and 126 it was found that PCB 153 has strong inhibitory effects in both acute and prolonged exposure, while PCB 126 is not a potent inhibitor. For illustration see Figure 5.

Figure 5: Influence of PCB 153 and 126 on GJIC by Machala, et al. (2003)

Part A: Fluorescent microphotographs of scrape-loading dye transfer assay in PCB 153 and PCB 126 treated cells

Part B: Documentation of a lack of GJIC recovery in PCB 153- treated cells.



They have also confirmed the fact that hepta up to octa-chlorinated congeners and non- *ortho* substituted PCBs elicite minimal or zero GJIC inhibition.

From 47 tested PCBs the most potent GJIC inhibitor was PCB 47 which IC_{50} was only 10.1 µM.

2.4. Degradation

The improper disposal and failures in implementation of conditions needed for the destruction of PCBs or even legally disposed waste could leak PCBs from landfills into the water and soil. This means that approximately 90 years of PCBs production have accumulated a wellspring of persistent environmental contamination that extends to animals and humans across the globe (Steinberg, 2007). Thus improper disposal and unintentional releases of these compounds led to the introduction of several hundred million pounds of PCBs into the environment, resulting in widespread environmental

contamination (Abramowitz, 1990).Therefore the abnormal chemically stable PCBs are present in all spheres of the environment and the solution for getting rid of these persistent compounds has been searched.

The success of the degradation is dependent on several aspects, i.e. structure of a given PCB, substituents and their position in the molecule, solubility, pH, temperature, presence of toxic and inhibitory substances, concentration (Borja, et al., 2005).

The possibilities nowadays are: thermal decomposition, physical- chemistry methods, chemical methods which are very expensive and there can be also a risk of creating more toxic compounds. Another and very effective possibility is biological degradation, e.g. microbiological transformation. However, the biodegradation is performed on pure cultures grown in the laboratories with a single congener. In nature, the degradation follows very complex metabolic pathways, including an interaction with other compounds and with various physiological and biological processes. There is a need to perform more "near-to-nature" experiments to optimize these processes (Urbaniak, 2007).

2.4.1 Biodegradation

Degradation in general is done through a cleavage of the ring and followed by dehalogenation. It includes both aerobic degradation and anaerobic degradation, it have been well documented and recognized as the major route of environmental degradation for these widespread pollutants (Higson, 1992). Another type how to biodegrade PCBs is also genetic degradation.

2.4.1.1. Limiting factors for biodegradation

There are several limiting factors for biodegradation to be overcome. It is type and degree of substitution and also the availability of PCBs in the aqueous solution. As they are lipophilic, PCBs are not easily soluble in the water. And this can cause a problem because it is important to be in contact with cells to produce the metabolites. To overcome this problem, there is a possibility of adding for bacteria non- toxic substances which increase the solubility. It can be for example Triton X-100 (Fetzner, et al., 1994).

As a limiting factor in anaerobic degradation could serve the presence of some

other acceptors of electrons which “consume” the electrons needed for the reductive dehalogenation (in our case dechlorination). These unwanted acceptors could be oxygen, nitric and sulfuric compounds (Vrana, et al., 1994).

2.4.1.2. Aerobic degradation

PCBs are in general degraded aerobically in two ways: by aerobic degradation as a growth substrate and by co-metabolism.

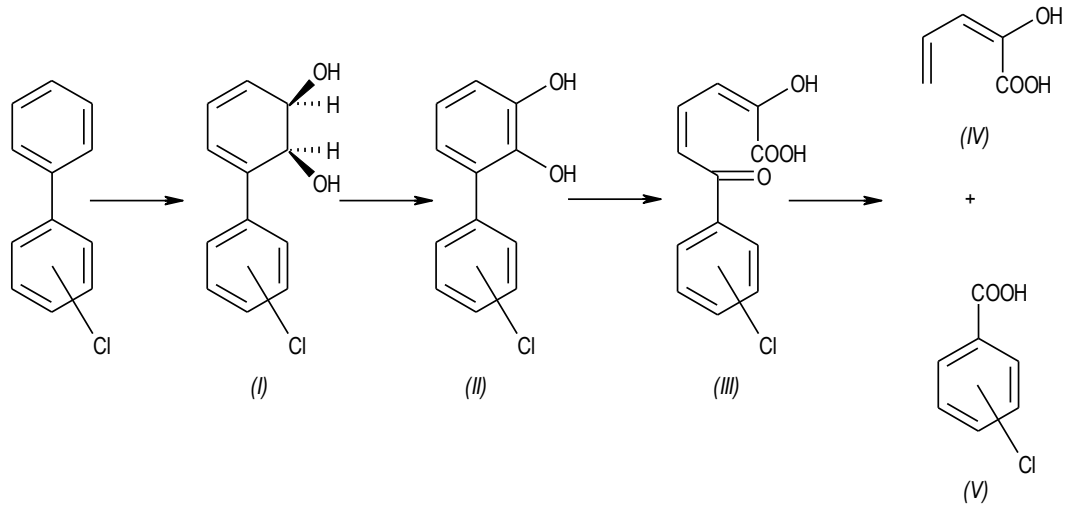
2.4.1.2.1. Aerobic degradation by bacteria

Aerobic degradation is sufficient for less chlorinated PCBs, whereas highly chlorinated congeners remain biorefractory to aerobic bacteria.
Aerobic degradation is a 4- step process starting with catalysis by biphenyl- 2,3- dioxygenase. It oxidizes unsubstituted positions (2, 3 or 5, 6), predominantly on the less substituted ring. The polychlorinated biphenyl is thus oxidized to 2, 3- dihydrodiol (I). After that, there is a dehydrogenation resulting in a formation of 2, 3- dihydroxybiphenyl (II). As a next step meta-cleavage at position 1 and 2 is performed resulting in 2-hydroxy-6-oxo-hexaphenyl-2,4-dienoic acid(III). Then the ring is cleaved and chlorobenzoate (V) and 2-hydroxy-2,4-pentadieneoic (IV) acid is formed.

This meta- cleavage can be done by bacteria *Pseudomonas, Vibrio, Aeromonas, Micrococcus, Acinetobacter, Bacillus,* and *Streptomyces.* Only requirement for this is that 2, 3- carbons are not chlorinated.

Various substrate specificity of biphenyl dioxygenase has great influence on the degradation of the congeners. This pathway for PCB degradation was worked out in the labs of Furukawa, in the early 70s. It is very effective for degradation of lightly substituted PCBs. For the scheme, see Figure 6.

Figure 6: Metabolic degradation of biphenyls and PCBs



2.4.1.2.2. Aerobic degradation as a growth substrate

It works for PCBs with the lower degree of chlorination, i.e. for mono-, di- or tri- substituted biphenyls (Bedard, et al., 1986). The bacteria, mostly Gram negative rods as e.g. *Pseudomonas cruciviae* (Takase, et al., 1986), are chosen according to their ability to grow on biphenyl or PCB agar. The PCB or biphenyl film is cleared around the colony.

2.4.1.2.3. Aerobic degradation by co-metabolism

Both bacteria able to grow on PCB media and fungi are able to degrade the PCBs. From fungi it is for example *Aspergillus niger* (Dmochewity, et al., 1988). This fungi has a cytochrome P-450 activity and it is able to attack lower polychlorinated PCBs. White-rot fungus *Phanerochaete chrysosporium* manages to attack lightly and moderately chlorinated PCBs but only in the very low concentrations (<500 ppb) (Thomas, et al., 1992).

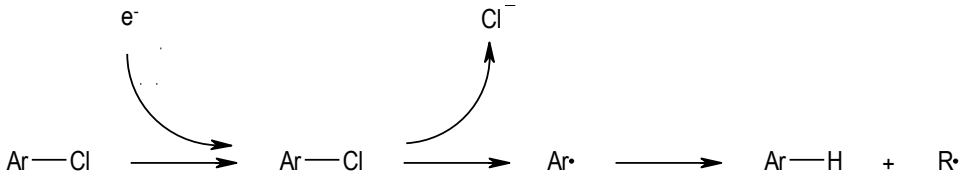
2.4.1.3. Anaerobic degradation

Anaerobic degradation is used for mineralization of higher chlorinated PCBs. Several studies were presented and they showed that substituents in *meta*- and *para*-positions are the best for dechlorination (Master, et al., 2002). Also Fava, et al., reported that dechlorination of Fenclor 54 primarily occurred in *meta*- and *para*- positions, while

ortho- substituted congeners accumulated in the medium.

Anaerobic degradation is done via reductive dechlorination and it is a 2-step process. The presence of electron donors is important. A chlorine atom is released as a chloride anion and it is replaced by a hydrogen atom (Fetzner, et al., 1994). Dechlorination is done mostly in *para*- and *meta*- positions, less frequently in the *ortho*- position (Brown, et al., 1987). In this reaction the structure of biphenyl ring remains the same. For the mechanism see Figure 7.

Figure 7: Reductive dehalogenation- dechlorination



2.4.1.3.1. Bacterial PCBs degradation genetics

Genes involved in the degradation of PCBs has been isolated from both Gram-negative (Ahmad, et al., 1990; Furukawa, et al., 1986), and Gram- positive bacterial species (Asturias, et al., 1993). These genes are able to encode PCB catabolism and they are clustered in an operon, called bphABCD. They are gained from e.g. *Pseudomonas pseudoalcaligenes* KF707 and *Pseudomonas* LB400. They have over 95% homology.

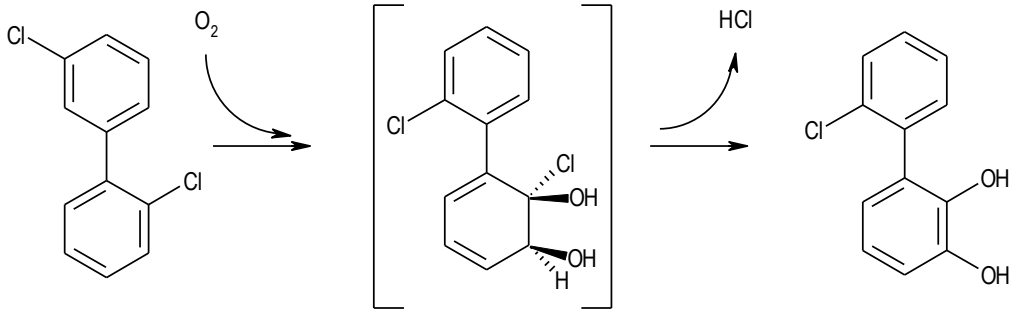
This type of degradation is good for highly chlorinated PCBs (up to and including hexachlorobiphenyl).

2.4.1.3.2. Ortho- PCBs and their biodegradation

In aerobic degradation, two atoms of chlorine in *ortho*- position increase the resistance to biodegradation. However, with the respect to congener's specificity of 3,4- biphenyldioxygenase there are different pathways of degradation. In this case, many di- *ortho* substituted congeners and some of tri- and tetra- substituted congeners are degraded faster. Thus, this enzyme is in some way able to attack *ortho*- chlorinated carbon.

From an unstable intermediate product (IV) a chloride ion is cleaved and 2, 3-dihydroxychlorbiphenyl is formed (VII). For the possible pathway see Figure 8.

Figure 8: Suggestion of 3, 4- dechlorination



(VI) *(VII)*

2.4.2. Other types of degradation

PCBs are very stable compounds and the biodegradation is not a fully sufficient or the fastest technique for their destruction. Thus also industrial techniques are used.

2.4.2.1. Thermal degradation

This type of PCB transformation leads to complete destruction. The temperature has to be above 700°C, otherwise even more toxic substances, e.g. polychlorinated dibenzofurans and dibenzodioxins are formed. This method is frequently used in industry (Sawhney, 1987).

Some factories and chemical plants may legally dispose of PCBs via flash-incineration at temperatures of at least 1200 degrees within a 2 second temperature ramp; however, failure to implement to these conditions properly can result in the formation of sister compounds with potentially greater toxicity than the PCBs themselves (Weber, et al., 2001).

2.4.2.2. Photochemical degradation

The photochemical degradation is one of the major processes to reduce PCBs in the environment.

The photochemical degradation usually reductively dechlorinates PCBs (also

examples of the isomerization and condensation of biphenyls were reported). For this kind of destruction, a UV source is needed. Very first laboratory experiments were done with mercury lamps with wavelength around 254 nm. This procedure showed dechlorination of PCBs. Other experiments were simulating sunlight- it was performed with UV radiation and UV fluorescent lamps. As a result, less chlorinated PCBs were obtained than in the initial material (Ruzo, et al., 1974).

It was found that less chlorinated PCBs decompose more slowly than higher chlorinated ones. For example, an exposure of PCBs at 310 nm reduces about 70% of tetra-, 96% of hexa- and 99% of octachlorobiphenyls (Urbaniak, 2007).

3. Basic determination of PCBs in the soil samples

Many analytical methods for detection and determination of PCBs are used nowadays. It is a result of a huge analytical method development and research done on these persistent compounds over the past 30- 40 years.

The procedure for successful determination has several pre-steps starting with sampling which has to be adapted to the type of sample. Samples can be divided to: water and aqueous samples, gaseous matrices i.e. mostly air, solid matrices (soils or water sediments) and matrices containing fat like human and animal tissues, as well as milk or blood. The analyses are very often expensive therefore some screening tests are also used. Then the sampling is followed by extraction and isolation depending on the source type, cleaning- up procedure and as a last step determination and evaluation of obtained data.

3.1. Screening tests

The analysis of samples with hypothetical toxicity are very often very expensive due to used chemicals, the time of preparation, i.e. pre-concentration, dissolution in proper solvents or finding the proper standards and their concentrations. Therefore screening tests are usually performed before the analysis.

Very fast and reliable technique is a bacterial bioluminescence test of toxicity (BBTT) which is discussed in more detail in part 4.2. Another possibility is the use of bioassay- the cells can originate for example from human or fish, and these cells were tailored to produce light in a dose-responsive way when exposed to some toxic compounds, in our case i.e. dioxins or dioxin-like compounds. The more toxic the sample is, the more light is emitted. Both mentioned types are good for fast determination of acute toxicity.

Moreover, the thin- layer chromatography can also be used, as well as mass spectrometry.

Screening tests are very accurate and reliable tests but they are not accurate to 100%. Thus, the combination of several screening tests is often used.

3.2. Sampling

3.2.1. Air sampling and extraction

Sampling PCBs from air is conducted using polyurethane foam or XAD resin (Integrated Atmospheric Deposition Network, 1994). Also silica gel, Florisil or glass beads can be used. The amounts of t air pumped through the sorbent must be very high, i.e. hundreds to thousands cubic meters. IADN (1994) specified this amount to the scale of 300 to 10,000 m^3. Then the sampling media is transferred to clean glass jars and stored at 4°C prior to extraction.

Extraction is done with various organic solvents, e.g. hexane, light petroleum. For these compounds Soxhlet or pressurized liquid extraction system is used in a clean laboratory environment (Integrated Atmospheric Deposition Network, 1994). Another possibility is dichloromethane or a mixture of some organic solvents as for example acetone and hexane (Larsson, et al., 1990). Another approach is to use supercritical fluid extraction (Hawthorne, et al., 1989).

3.2.2. Water sampling and extraction

This type of sampling is in general more difficult due to the low concentrations of PCBs in environmental waters. Therefore large volumes are needed- in general liter up to hundred of liters.

Extraction is done directly with water-immiscible organic solvents, i.e. hexane or dichloromethane. The last mentioned one was used in liquid-liquid extraction in a continuous flow approach (Neilson, et al., 1988). It involves a one pass through extraction unit where the sample is metered into a mixing chamber with this solvent and flowed out to an overflow exit. There is however a disadvantage to this method, namely a high risk of contamination from surrounding air.

Another possibility is the capture on a solid sorbent like on Tenax, XAD- resin or polyurethane foam. Polyurethane foam was used for example by Petrick, et al. (1996) who described this method as very sufficient for determination of PCBs at very low

concentrations (pg/L). XAD- resin was used by Achman et al. (1993) who in his study pumped lake water (~ 100 L) directly into large- diameter filters (0,7 µm nominal pore size) and than through these XAD-resin columns.

This leads to the conclusion that sampling and extraction joined to one single step are very sufficient and moreover they do not need any additional clean-up.

The results can be affected by pH. It was found that acidic or neutral extractions may lead to an underestimation of PCB concentration in water (Maguire, et al., 1989).

3.2.3. Soil sampling and extraction

Soil samples, in this case sediments, are first of all homogenized and dried if needed. The extraction is done with a mixture of acetone and a light aliphatic hydrocarbon in a Soxhlet apparatus, a separation funnel or an ultrasonic bath. As another medium, hexane, acetone or ethyl acetate can be used, also isopropyl alcohol and dichloromethane or methanol and dichloromethane used consecutively.

3.2.4. Human and animal tissue sampling and extraction

First the PCBs are extracted together with the fat and then in the following step they are separated. Animal tissue is homogenized and dried with anhydrous sodium sulfate and the extraction is done again with hexane or light petroleum. Usage of dichloromethane, benzene or cyclohexane is also possible. Often mixtures of solvents, e.g. a small amount of diethyl ether in light petroleum (Mauck, et al., 1977) or benzene-acetone mixture (Mes, et al., 1990), hexane- acetone (Goerke, et al., 1990) or toluene-ethyl acetate mixtures are used as well. Very often used method is also a saponification of the fat and then a light aliphatic hydrocarbon is used for the extraction (Tanabe, et al., 1987).

The most important fat- containing liquid matrices are blood and milk. From blood, the determination of PCBs is done both directly from blood and from blood serum. In both usual organic solvents are used, e.g. hexane, acetone- benzene or acetone- hexane. Very fast technique is an extraction using conventional solvent partitioning from blood plasma lipids. A mixture of ammonium sulfate/ethanol/hexane (1:1:3) is used (Bjerregaard, et al.,

2001).

From milk PCBs can be extracted using conventional lipid extraction techniques involving liquid–liquid extraction with hexane/acetone (Burke, et al., 2003). Another technique is a blending with acetonitrile and potassium oxalate (AOAC, 2003), followed by C18- solid phase extraction (Focant, et al., 2003).

3.3. Purification

Purification procedure is used for removal of interfering substances. Mostly liquid-solid adsorption chromatography, gel permeation chromatography and chemical methods are used. Less frequently a removal of fat by low-temperature precipitation or liquid-liquid partitioning is used.

3.3.1. Liquid- solid adsorption

3.3.1.1. Common adsorbents

Separation of PCBs can be relatively directly done for soil, sediments and vegetation samples. These samples contain low amounts of fat. For this purpose silica gel or Florisil adsorption columns can be used. The main purpose in this step is to separate non-polar PCBs from more polar ones and also to remove substances which can interfere in the determination, i.e. which can have the same retention time etc. This clean-up procedure is done with an extract in a small volume of apolar solvent. The solution is fractionated by elution with hexane and followed by one or two other elutions to increase the polarity of the solution. As a result, polar compounds stay in the column. These columns are effective for clean-up of water, sediments, pigments, wood, oils, plant and animal tissues and blood as well as blood serum or paper.

Alumina can be used as well, but there is a risk of small losses due to dehydrochlorination of some PCBs on alumina (Muir, et al., 2006). Alumina is used for clean-up of extracts from air, sediments, animal tissues, milk or blood.

3.3.1.2. Size- exclusion columns

Size- exclusion columns are used for samples with high lipid contents (>10%). The separation can be achieved using size-exclusion or gel permeation chromatography (GPC). Both mentioned types remove the fat from extracts.

The gel widely used is BioBeads- SX3. It is a neutral porous styrene divinylbenzene resin with size exclusion of 400 Da (Ribick, et al., 1982). Advantage of GPC is that it is not destructive and columns can be reused. On the other hand there is a requirement for large volumes of purified solvents or expensive HPLC columns. Because the fat is not removed completely and it is needed to remove low molecular weight lipids, Florisil or silica gel is used. As eluants mostly mixtures are used, e.g. cyclohexane/ ethyl acetate, toluene/ethyl acetate and so on.

3.3.1.3. Lipid destruction

Possibly interfering fats can be removed by lipid destruction. Lipid destruction is done using sulfuric acid- the sample can be washed or extract is eluted through sulfuric acid in KOH-treated silica columns or just silica columns. Ethanolic- KOH treatment of Soxhlet extract can be used as well (Muir, et al., 2006). An adsorption column fractionation on silica or Florisil is then used for extracts.

3.3.1.4. Activated carbon

Activated carbon makes a separation of PCBs according to their polarity possible. It does not degrade them. The advantage of an activated carbon is it has very high affinity to adsorbate (i.e. PCBs in this case) with high molecular weight and low solubility. The only requirements which should be taken in account are the proper pore size, concentration and right fluid stream. It is a useful method how to decrease PCB´s contamination in groundwater, sediments and therefore decreases the uptake by living creatures in the environments such as earthworms and clams (Werner, et al., 2005).

3.4. Measurements

For the quantification of PCBs several methods can be used. It can be gas chromatography (GC), high- performance liquid chromatography (HPLC), Fourier transform infrared spectrometry (FT-IR), radioimmunoassay etc. Some of them can be of course coupled to obtain more precise results. Nowadays, the most sufficient and the most used method is gas chromatography often coupled with mass spectrometry (MS) or with electron-capture detection (ECD). Between GC advantages belong sensitive detectors, efficient separation and good reproducibility.

3.4.1. Gas chromatography

Separation by GC has several aspects to which attention should be paid, i.e. proper injection which minimizes analyte band- broadening, good choice of carrier gas, optimized velocity of gas carrier, GC oven programming, proper column dimension such as number of plates, inside diameter, length, film thickness as well as type of stationery phase used (Cohran, et al., 1999).

3.4.1.1. GC Injection

The injection is crucial for the GC analysis. If packed columns are used there usually is not any problem. In a case of capillary columns the combination of split/split less injection system and on- column injection is performed. This coupling is performed due to the possible discrimination of some PCBs of split less injection due to the sharp range of PCBs boiling points and also due to possible degradation of labile compounds. On the other hand the on-column injection is very sensitive to any dirt, therefore it requires highly clean extracts to avoid matrix effects. These effects could be overcome by retention gaps and moreover, it makes the injection of larger volumes of samples possible.

3.4.1.2. GC columns

Two types of columns are mostly used for the analytical quantification of PCBs, especially for *ortho*- substituted PCBs, packed columns and capillary columns. Non-polar or slightly polar stationery phases are used.

The disadvantage of packed columns is that each peak represents several congeners. Therefore capillary columns are predominantly used, which allow identification of the individual congeners. The basic technology for separation of PCBs in this way did not change very much from its first publication – for the first time, 5% phenyl methyl silicone phase and a long temperature program about 100 minutes long was used (Mullins, et al., 1984). High- resolution separation of PCBs was achieved.

Columns 25-65 m long are used, mostly 60 m. The inner diameter (I.D.) is in the rangeof 0.2-0.32 mm. The carrier gas is predominantly hydrogen because of its best peak resolution and efficiency. Elution of *ortho* PCBs is dependent on their substitution. The biggest one are PCBs with four *ortho*- substitutions, then with three, two, one and as a last one with no *ortho*- substitution.

3.4.1.3. GC separation

Besides the commonly used techniques also methods with respect to some specific properties can be used. For example many PCBs are optically active or chiral isomers. 19 of PCBs are so- called atropisomers, i.e. compounds with three or four ortho substituted positions which are very stable to racemization which are also chiral. Chiral analysis can be done by GC by using chiral capillary GC columns, for example cyclodextrins chemically bonded to a polysiloxane. This phase is relatively stable and low bleeding but it is not a part of routine PCB analysis. On the other hand it is not difficult to implement this to frequently used gas chromatography- electron capture detection (GC- ECD) or gas chromatography- mass spectrometry (GC-MS) analysis.

The comparison of possible separation is demonstrated in Table 4.

Table 4: General guidance on GC analysis and data reporting for PCBs by (Muir, et al., 2006)

GC detector	Analytes	Configuration	Advantages/disadvantages	Detection limits
Capillary GC- with electron capture detection	All *ortho*-substituted PCBs	30 or 60m x 0.25 mm I.D. Column with H_2 carrier gas. Dual column and intermediate polarity columns	Inexpensive and easy to operate. Good sensitivity for all POPs. Adequate for routine tasks.	Ng/g levels; HCB ~ 0.5 pg
			High potential for misidentification of some POPs due to coeluting peaks.	
Quadruple mass spectrometry in electron ionization (EI) mode	All PCBs	30 m x 0.24 mm I.D. low- bleed columns with He carrier gas. Selected ion mode for target POPs.	More complex to moderate and maintain. Newer instruments have adequate sensitivity for routine POPs monitoring at low pg/µL conc. Much less potential for misidentification than with ECD.	HCB ~ 1-10 pg
			Moderately expensive.	
Quadruple mass spectrometry in electron capture negative ionization (ECNIMS) mode	PCBs with >4 chlorines	30m x 0.25 mm I.D. low- bleed columns with He carrier gas. Selected ion mode for target POPs.	Comparable sensitivity to ECD in SIM mode for some POPs. Much less potential for misidentif. than with ECD.	HCB ~0,1 pg
Ion trap mass spectrometry using MS/MS mode	All PCBs	30m x 0.25mm I.D. low-bleed columns with He carrier gas. Same columns as quadruple MS.	Comparable sensitivity to ECD in MS/MS mode for some POPs. Much less potential for misidentification than with ECD.	HCB ~ 1 pg
High-resolution magnetic sector mass spectrometry in electron ionization (EI) mode	All PCBs	30m x 0.25mm I.D. low- bleed column with He carrier gas. Selected ion mode for target POPs at 10,000x resolution	Comparable sensitivity to ECD in SIM mode. Highly reliable identification at low pg/µL	HCB ~ 0,05 pg

4. Determination of occurrence of *ortho-* substituted PCBs in a soil sample

4.1. Soil sampling

The soil sample was collected near by the Spolana Neratovice about 10 meters away from the gate. The sample consisted of a soil from the deep of 20cm. The soil was afterwards put into dark glass and stored in 4°C.

4.2. Screening

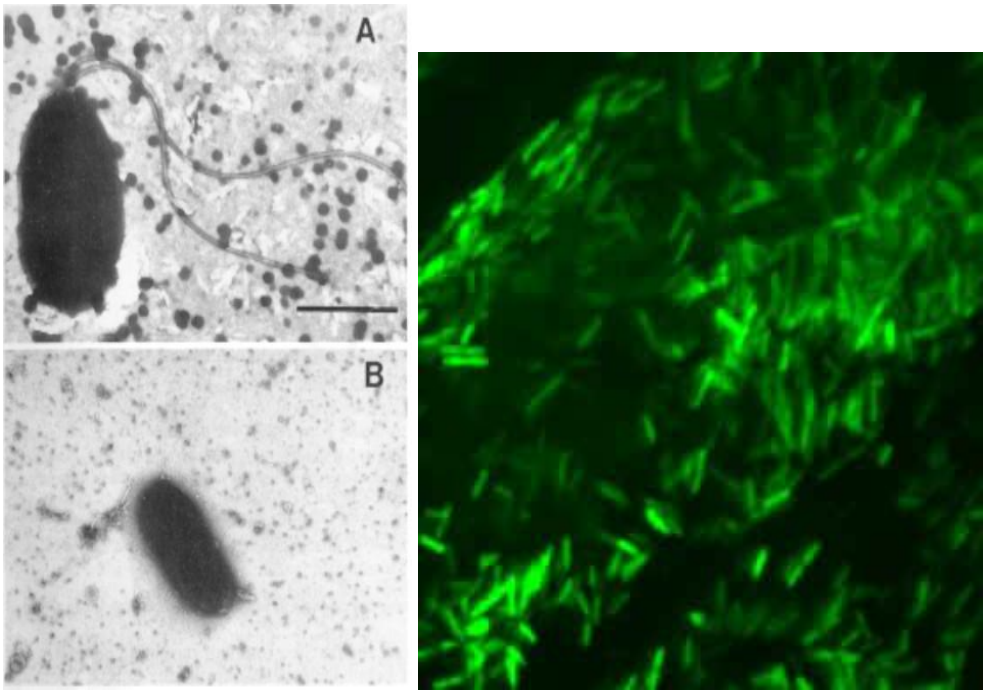
As a pre- test, i.e. testing if some toxic compounds are present in the sample, the BioTox™ Flash Test was used. The test was performed in the Research Centre for Toxic Compounds in the Environment (Recetox), in the independent department at Faculty of Science, Masaryk University in Brno.

BioTox™ Flash Test belongs to a group of Bacterial bioluminescence tests of toxicity (BBTT). These tests are based on an ability of some organisms to bioluminesce. There is a measure of inhibition of bioluminescence (produced by bacteria) with the respect of addition of the sample tested. This inhibition informs about real complex toxicity of the sample, not about the exact compounds which are present in the sample. The tests can be used for a detection of highly toxic compounds which are not detected by normally applied methods. They are very good for screening because they are fast, very sensitive, cheap, reproducible and small volumes of sample are used. Another advantage of this kind of testing is that it can be used for testing of toxicity in water, sediments, soil and air (Broers, et al., 2004). On the other hand the tests can be influenced by colored samples (e.g. by soil) as the bioluminescence can be masked by the color. For this kind of samples kinetic method are used instead.

For BioTox™ Flash Test *Vibrio fischerie* is used, according to International organization for Standardization (ISO) 11348- 3 (ISO, 1998). Nowadays, there is an optimization with *Photorhabdus luminescence*. *Photorhabdus luminescence* is a soil

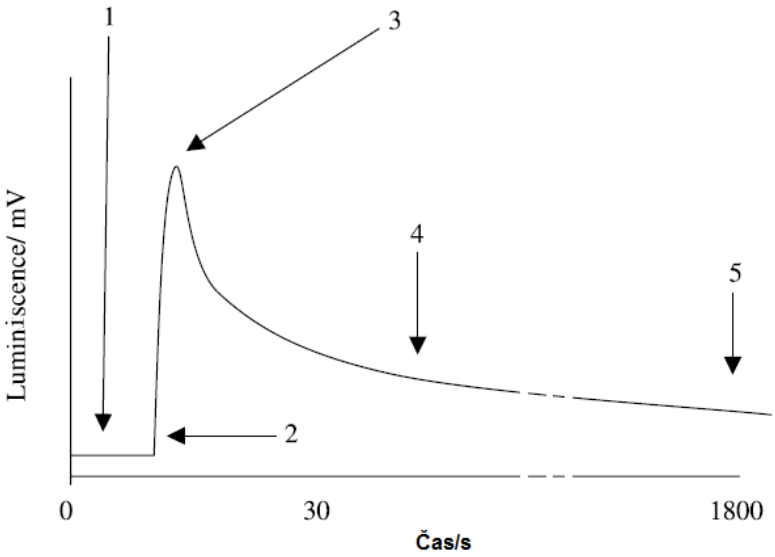
bacterium on contrary to *Vibrio fischerie* which lives in the sea and therefore high concentrations of salts have to be added to the growing medium. See the picture of *Vibrio fischerie* and in *Photorhabdus luminescence* Figure 9.

Figure 9: Vibrio fischerie (source: web.uconn.edu/mcbstaff/graf/VfEs/VfEssym.htm) on the right; Photorabdus luminescence on the left (source: staff.bath.ac.uk/bssnw/photor14.jpg)



It is believed that the light produced by non-stressed bacteria is constant or it can slightly decreases till time of 1000 seconds and that a production of light decreases immediately after addition of toxic compounds. Therefore this test starts at time of 0 seconds when the signal refers to the contact with the sample. At this time an influence of toxic compounds is not expected but only the influence of the matrix, e.g. color. Another measure is done after 30 seconds. The ratio gained from these values is used as a measure of toxicity. For the illustration see Figure 10.

Figure 10: Kinetic test of toxicity with bioluminescence bacteria: 1- start of measure, 2- injection of bacterial suspension, 3- report of the maximum value of luminescence at time t= 0-2s, 4- report of value of luminescence at time t= 30s, 5- report of signal at time t= 30 minutes; shaking during the whole procedure; according to (Rusova, 2010).

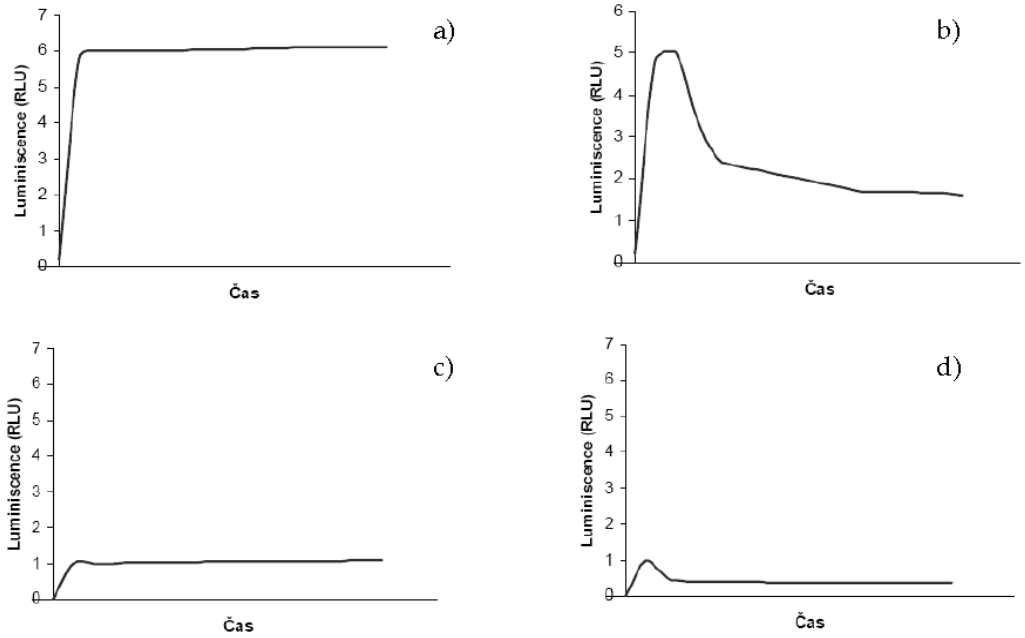


As a reference the value of the maximum luminescence of non- stressed bacteria is used immediately after addition of bacteria to the sample. An influence of turbidity and color is respected during the whole measurement (Lappalainen, et al., 2001).

Accordingly to the shape of the curve, we can distinguish if the sample is toxic or not. In the non toxic samples there is high luminescence after injection which remains constant after 30 s. The environment for the bacteria is not conflict and it does not disrupt the biochemical process, i.e. for example the bioluminescence. In toxic samples, the starting value is relatively high but it is not the same as in the non- toxic samples. After 30 s the intensity of luminescence has gradually decreased.

There is also influence of turbidity which should be taken into account. For the shapes of curves see Figure 11.

Figure 11: Comparison of curves of toxic and non- toxic responses- a) Clear non- toxic sample, b) clear toxic sample, c) turbid non- toxic sample with absorption effect, d) turbid toxic sample with absorption effects.



4.2.1. Preparations for screening test

4.2.1.1. Bacteria

Photorhabdus luminescence was used for the experiment. It was stored in 15°C in a solution of trypton soya broth (TSB), phosphate buffer saline (PBS) and glycerol.

4.2.1.2. Soil sample

The sample was lyophilized overnight to dry it completely. The soil were homogenized in a mortar and then sowed. 140 mg of the sample was weighted (m_{EXACT} = 145,8 mg). 1.4 ml of 1% PBS was added to the sample and vortexed. Therefore the highest concentration was 1.4 mg/ml.

A microtitration plate was used for the experiment. Positive control, negative control and diluted solutions were applied to the plate in doublets. The dilutions of samples were performed directly on the plate and it was done with 1% PBS. The dilution factor was 1,

i.e. 40 µl of the sample from dilution row to 40 µl of 1% PBS. Each sample was mixed by pipetting 10 times. For illustration see Figure 12.

Figure 12: Distribution of the samples on the microtitration plate with the arrows symbolizing the dilution in the dilution raw; NC- negative control; PC- positive control

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC	NC	PC	PC								
B	NC	NC	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5
C	NC	NC	S6	S6	S7	S7	S8	S8	S9	S9	S10	S10
D												
E												
F												
G												
H												

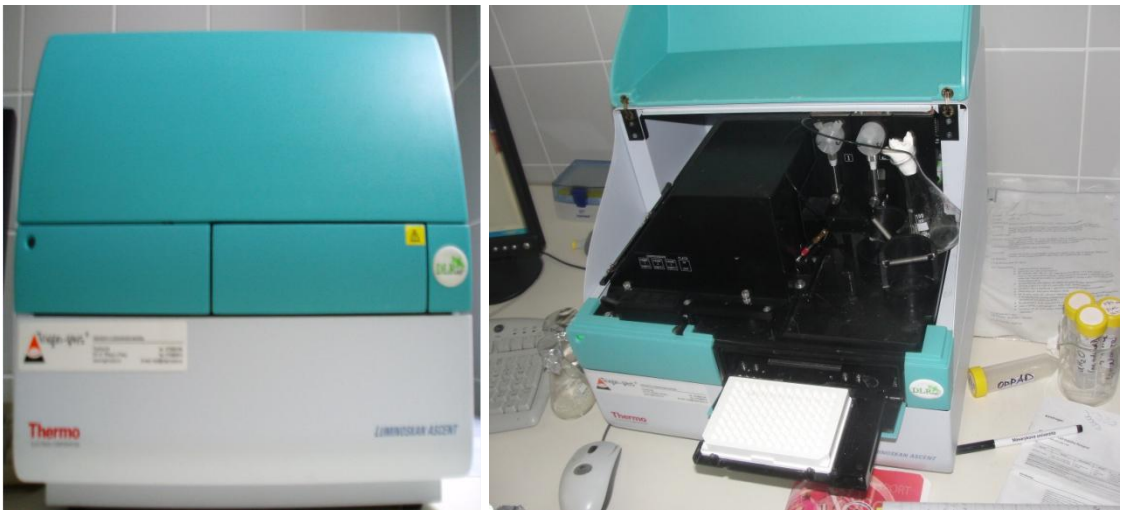
As a negative control, 1% PBS was used to control the right luminescence of bacteria, as a positive control $K_2Cr_2O_7$ was used. For both the volume of 80 µl was used.

4.2.1.3. The measurement

The measurement was performed on the luminometer LuminoSkan Ascent (with dosing system for injection of bacterial suspension to the samples, Ascent software version) (Figure 11).

Before the measurement, the instrument was repeatedly washed 5 times with distilled water, then with PBS and finally with TSB. It was done to create the same environmental conditions in which the bacteria were used to live, and also to prevent some stress situation which could influence the results of the measurement. After the cleaning, the house- pipe was left in the bacterial suspension. The temperature was 30°C.

Figure 13: Luminometer LuminoSkan Ascent



Entire measurement was done in three major steps. First of all, there was an injection of bacterial suspension to the samples to measure the bioluminescence at time t= 0-2 s. Secondly, the solution was mixed and thirdly, the bioluminescence at time t= 29-31 s was measured. All three steps were performed for each of the measured holes. Parameters of the measurement are summarized in Table 5.

Table 5: Parameters of the measurement by luminometer

Parameters:	
Temperature	30°C
Injection and measurement of bioluminescence at time t= 0-2 s	
Volume of injected suspension	20 µl
Integration time	40 ms
Number of measurement	32 x
Mixing	
Whole time of mixing	25 s
Speed	1200 rpm
Measurement of bioluminescence at time t= 29- 31 s	
Integration time	40 ms
Number of measurement	34 x

4.2.1.4. Data evaluation

Results of the measurement were evaluated by a protocol created in Office Excel 2007. The inhibition of the bioluminescence and identification of 50% effective concentration (EC_{50}) was calculated by GraphPad Prism version 5.0.

How the results were exactly calculated is above the framework of this thesis. Therefore just the obtained data are presented.

Table 6: Results- the "+" sign symbolizes an inhibition of bioluminescence, the "-"sign symbolizes a stimulation of bioluminescence

Hole number	Inhibition/stimulation	Results in percent (%)
B1	Negative Control	
B2	Negative Control	
B3	0,216	21,593
B4	0,216	21,561
B5	0,065	6,477
B6	0,056	5,555
B7	-0,001	-0,061
B8	0,088	8,815
B9	-0,004	-0,366
B10	0,026	2,621
B11	-0,006	-0,634
B12	-0,014	-1,356
C1	Negative Control	
C2	Negative Control	
C3	-0,116	-11,636
C4	-0,106	-10,645
C5	-0,077	-7,742
C6	-0,057	-5,724
C7	-0,048	-4,835
C8	-0,038	-3,773
C9	-0,057	-5,738
C10	-0,093	-9,333
C11	-0,055	-5,492
C12	-0,058	-5,775

4.2.1.5. Interpretation of results

Negative values mean that there was a stimulation of the bioluminescence and the positive values mean there was an inhibition of the bioluminescence. From the results we can see the biggest inhibition was caused by samples B3 and B4. These samples were the highest concentrated samples used. Their inhibition was converted to percent and it was found out that the inhibition they caused was ~21%. This tells us that the sample is very slightly toxic because samples which inhibition is lower than 20% are said not to be toxic.

From this test we cannot say anything about the compound or compounds which causes this very low inhibition of bioluminescence. Therefore other additional and suitable analyses have to be performed.

4.3. Identification of unknown toxic compounds by Gas chromatography with electron- capture detection (ECD)

For electron- capture detection (ECD), a 5890 Series II Gas Chromatograph was used with the following parameters:
Column: Rtx-1614, 15 m, ID 0.25 mm, 0.1 µm film thickness
Injection Volume: 1 µl of the sample
Carrier gas: helium, inlet pressure 150 kPa
Temperature: starting temperature 60°C for 1 min, then gradient 15°C/min to 200°C, then gradient 7.5°C to 275°C, 12 min at this temperature.

4.3.1. Preparation of the standard and sample solution

4.3.1.1.Standard preparation

As a standard solution, the mixture of 6 PCBs was used (Dr. Ehrenstorfer-Schafers). Product identification was 20030100 PCB-mix. The list of PCBs congeners is given in the Table 7.

Table 7: PCBs contained in the mixture

PCB mixture by Dr. Ehrenstorfer- Schafers	
PCB 28	PCB 138
PCB 52	PCB 153
PCB 101	PCB 180

Starting concentration was 10 mg/l. The standard calibration solutions were prepared by dilution of the stock solutions with toluene for all PCBs to the following concentrations: 0.1 ng/µl, 0.2 ng/µl, 0.5 ng/µl, 1 ng/µl and 2 ng/µl.

4.3.1.2. Sample preparation

Soil sample was homogenized and seven samples were prepared. Four samples were prepared for the analysis, therefore about 10 grams of the soil was weighted into the vials. As a solvent, 35 ml of hexane- acetone mixture (3:2) was used. The extraction was performed by an ultrasonic bath for 10 minutes. Then the extraction solutions were separated from the solid sample which was again extracted by 35 ml of hexane- acetone mixture. This step was repeated till 4 extractions were performed. The extracts were filtered through glass wool and Na_2SO_4. The combined extracts were transferred into pear- shaped vials. Evaporation was performed by nitrogen flow at the temperature of 30°C, till the hexane- acetone mixture was completely evaporated. Then 1 ml of toluene was added and the solution was vortexed. The sample was too concentrated therefore the dilution was diluted to 5 ml with toluene.

Three samples were prepared for determination of the water content, 7 grams into three vials were weighted and then they were left in an oven for 2 hours at 110°C. For exact amounts see Table 8.

Table 8: Exact amounts used for preparation of sample solutions

Sample for the analysis	Mass [g]
Sample 1	10,032
Sample 2	10,008
Sample 3	10,046
Sample 4	10,041
Sample for determ. of water content	
Sample 5	7,032
Sample 6	7,129
Sample 7	7,018

4.3.1.3. Results

4.3.1.3.1. Dry residue

After one hour of drying at 120°C, the following results were obtained, summarized in Table 9.

Table 9: Content residues results [%]

	M $_{\text{before drying}}$ [g]	**M $_{\text{after drying}}$ [g]**	**Content of dry residue**	**Content of dry res. in %**
Sample 5	7,032	5,39	0,766496	76,6
Sample 6	7,129	5,43	0,761678	76,2
Sample 7	7,018	5,386	0,767455	76,7

4.3.1.3.2. Calibration curves

Peak areas are given in the Table 10. For the shortness, the equations and correlation coefficients of standards are given in the Table 11. PCB 153 eluted earlier then PCB 138. The spectra of standards see in Figure 14.

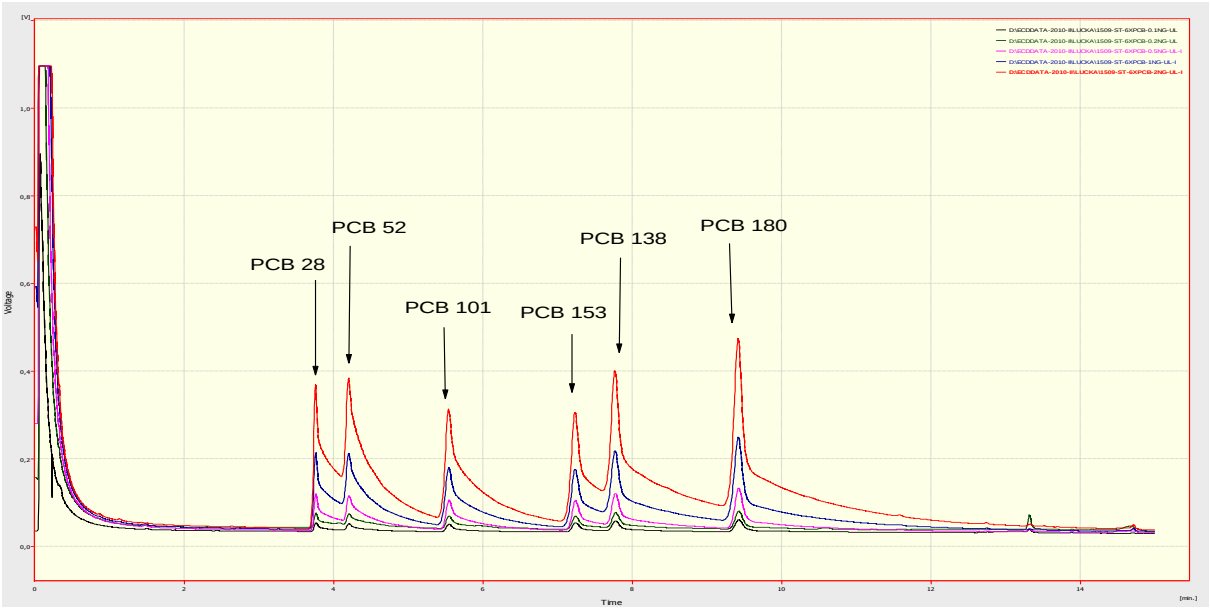
*Table 10: Peak areas of calibration solutions [mV*s]:*

Peak areas[mV*s]	PCB 28	PCB 52	PCB 101	PCB 153	PCB 138	PCB 180
0,1 ng/l	130,656	85,249	217,9	177,839	249,082	172,191
0,2 ng/l	287,236	331,384	527,983	379,145	670,001	351,756
0,5 ng/l	616,979	701,653	1179,316	588,103	909,021	614,628
1 ng/l	1291,224	1521,316	2633,047	1173,484	2011,345	1171,965
2 ng/l	2395,51	2953,982	5101,716	2167,209	4087,088	2410,701

Table 11: Regression equations and correlation coefficients:

PCBs	Regression equations	Correlation coefficient
PCB 28	y= 0,0008x - 0,0216	R^2= 0,9984
PCB 52	y= 0,0007x + 0,0086	R^2= 0,9988
PCB 101	y= 0,0004x + 0,0074	R^2= 0,9991
PCB 153	y= 0,0009x – 0,0712	R^2= 0,9949
PCB 138	y= 0,0005x – 0,0239	R^2= 0,9937
PCB 180	y= 0,0008x – 0,035	R^2= 0,9975

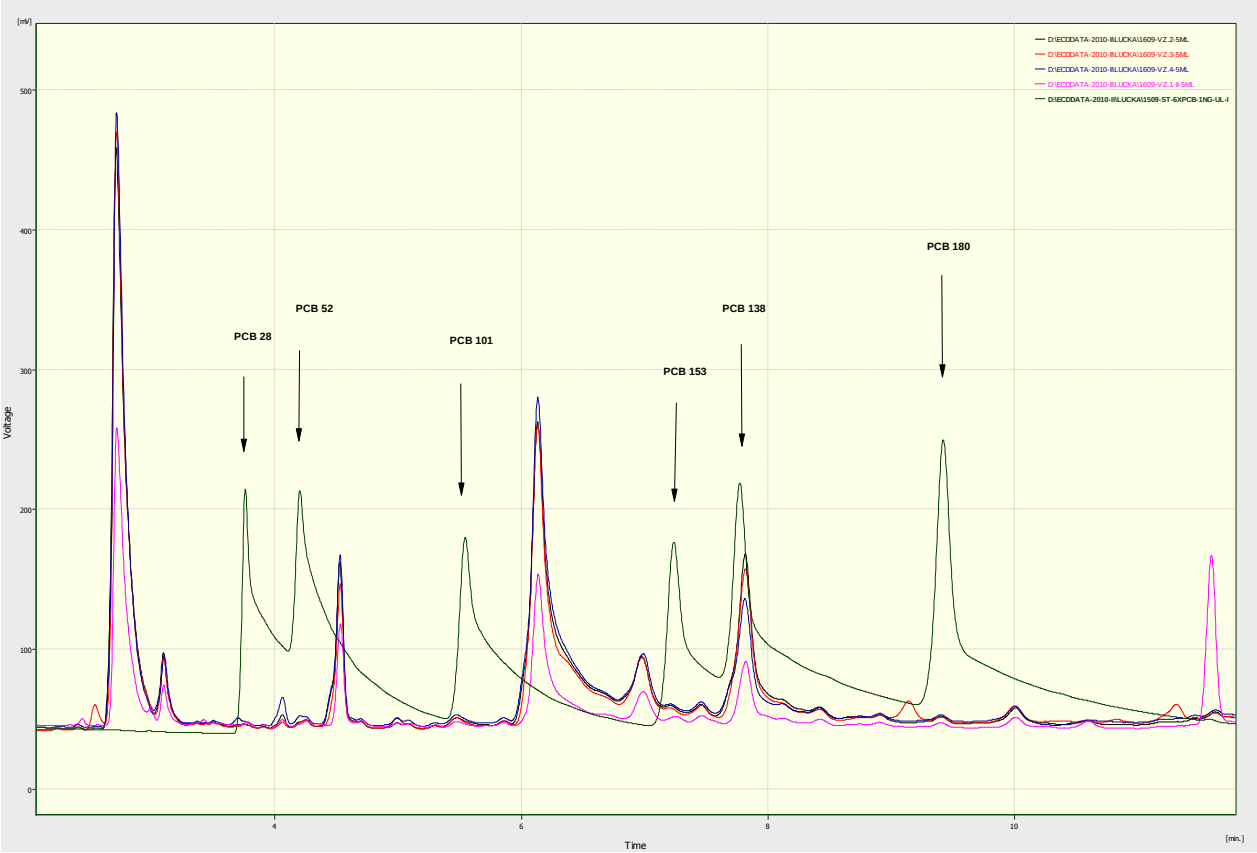
Figure 14: Standard solutions with the increasing concentrations, starting from 0,1 ng/µl, the highest concentration was 2ng/µl.



4.3.1.3.3. Samples

The measurement was performed for each of four samples. Except PCB 138, no other PCBs were detected. See the chromatogram in Figure 13, as standard the solution with concentration 1 ng/µl was used (black line).

Figure 13: Chromatogram of samples and standard solution



Peak areas of PCB 138 are summarized in Table 12.

Table 12: Peak area of the PCB 138

Peak area [mV*s]	Sample 1	Sample 2	Sample 3	Sample 4	Average
PCB 138	794,994	1295,9867	1204,527	941,509	1059,254

From the peak areas measured by ECD, the calculation of the concentration of PCB 138 could

be performed using the calibration equations from the standard calibration curves. The results are given in Table 13.

Table 13: Calculated concentration of PCB 138 per ng/µl, ng/5ml and per gram of dry residue

Concentration	**ng/µl**	**ng/5ml**	**ng/$g_{dry\ residue}$**
Sample 1	0,374	1870	141,667
Sample 2	0,624	3120	236,931
Sample 3	0,578	2890	218,634
Sample 4	0,447	2235	169,166
Average	**0,506**	**2530**	**191,672**
Standard deviation	0,100	577,442	38,021
Confidence interval	0,098	565,883	37,260
Relative stand. deviation	19,766	22,824	19,836

4.4. Gas Chromatography- Mass Spectrometry (GC-MS)

To confirm the results obtained from gas chromatography with electron- capture detection (GC- ECD), GC- MS was used.

For GC-MS measurements ITQ 1100 instrument was used with the following temperature gradient. As a solvent, toluene was used.

Column: ZB-5, 30 m, ID 0.25 mm, film thickness 0.25 µm

Injection Volume: 1 µl of the sample, splitless

Carrier gas: helium, inlet flow rate 1 ml/min

Temperature: starting oven temperature 100°C for 3 min, then gradient 10°C/min to 220°C, then gradient 2°C to 260°C, then 5°C to 285°C, 8 min at this temperature.

4.4.1. Analysis by GC-MS

GC- MS is less sensitive method compared to GC-ECD for halogen containing compounds. On the other hand, it is a good technique how to support or deny the results obtained by other methods and moreover, it can show and identify other than halogen containing compounds present in the sample. The GC- MS TIC (Total Ion Current) chromatogram of the standard solutions can be seen in Figure 14. The TIC chromatogram of the analyzed sample is in Figure 15.

Figure 14: GC- MS TIC chromatogram of standard solutions

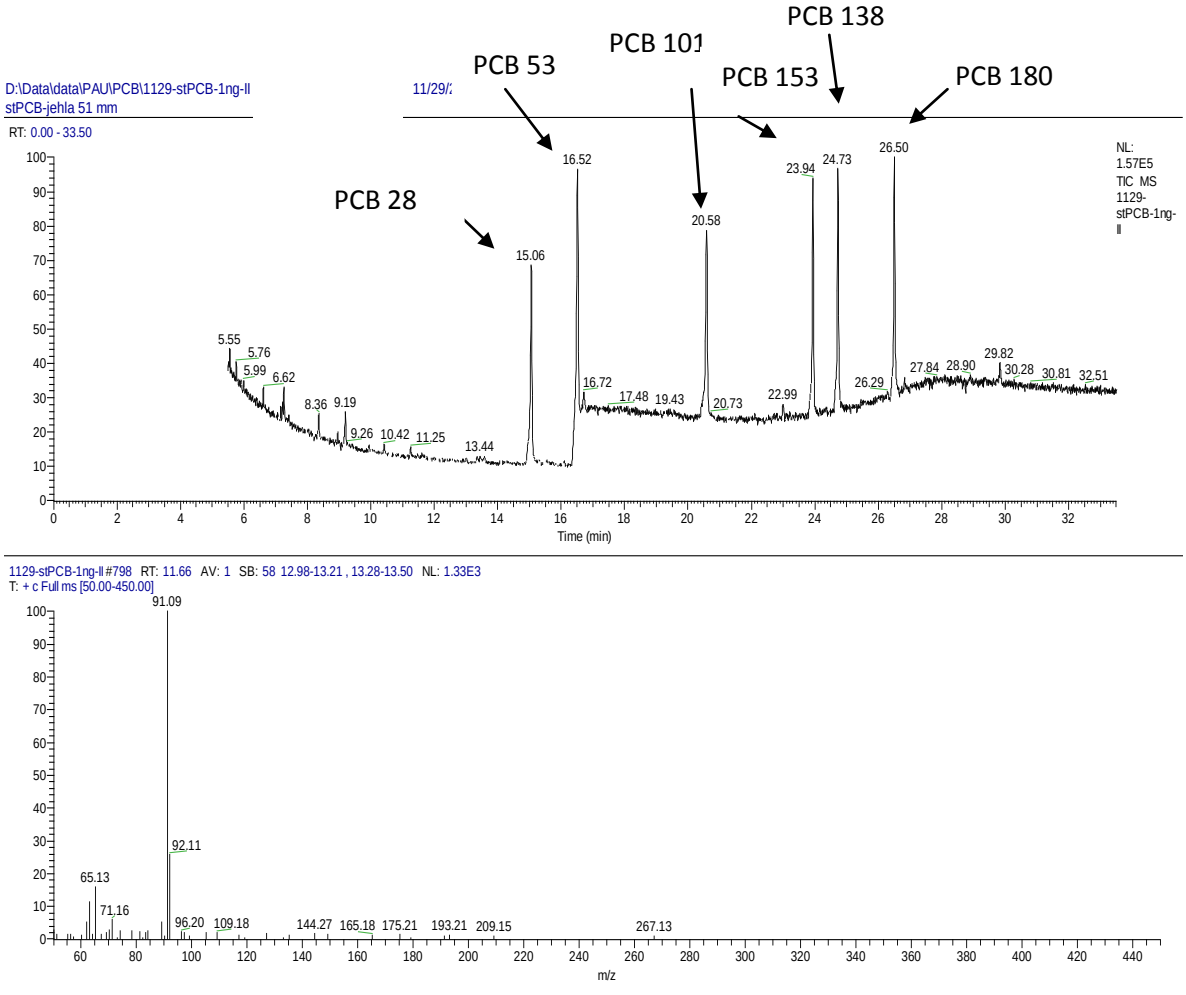
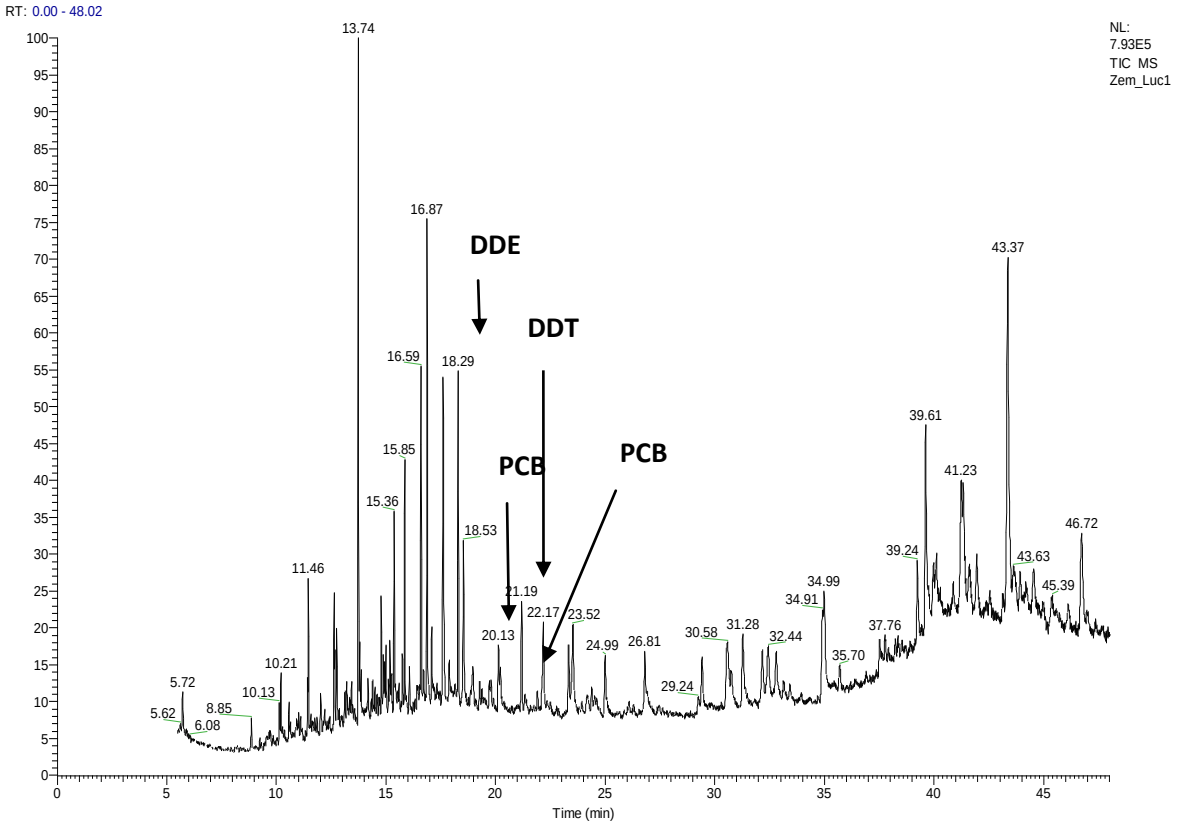


Figure 15: GC-MS TIC chromatogram of the analyzed sample



Discussion

Ortho- substituted PCBs were thought to have very low biological activity. In the latest studies it has been proved they are as dangerous as other PCBs or even more. Thanks to their structure they easily bioaccumulate in nature, they have antiestrogenic effects (Oh, et al., 2007), they can interrupt a communication between cells (Machala, et al., 2003) and they can also be carcinogenic. Therefore, the thesis is focused on this interesting topic.

From the literature search it is clear that the effects of these, in the past industrially produced chemical compounds, but still present in the environment, should not be underestimated and more studies should be done in this field to find out all possible effects in the environment, especially the bioaccumulation and influences on living organisms. The literature search has also shown that the way to handle and safely destroy PCBs is not fully determined.

The practical part of the thesis was set to analyze a soil sample collected near Spolana Neratovice chemical plant. PCBs as well as other compounds, i.e. dioxins, were present here and still are in detectable amounts. The sample was collected on chance, without any information about the possible occurrence of these compounds in the near area of Spolana chemical plant.

At first, the sample was screened for any possible toxicity using bioluminiscence bacteria. This experiment was performed in Recetox, an independent department at Faculty of Science, Masaryk University in Brno which focuses on environmental contaminants, especially persistent organic pollutants (POPs), polar organic compounds, toxic metals etc. To say the compound is toxic, the inhibition of the bioluminescence has to be more than 20% in the test. The sample collected nearby Spolana Neratovice inhibited the bioluminescence by 21%, but the inhibition is a matter of the concentration.

To estimate if the compounds in the soil sample which showed a very slight toxicity are the compounds of our interest, additional analytical techniques were used, i.e. GC-ECD and GC-MS.

For preparation of standards, PCBs frequently found in the environment, congeners Nos. PCB 28, 52, 101, 138, 153 and 180, called indicator congeners, were used. The standard calibration solutions were prepared by dilution of each PCB solution with toluene to 0.1

ng/µl, 0.2 ng/µl, 0.5 ng/µl, 1 ng/µl and 2 ng/µl. The peaks were clear and fully visible in all concentrations, therefore we can say the sensitivity of the GC-ECD was sufficient. The detection limit was not exactly measured due to lack of time but according to the lowest concentration, which was 0.1 ng/µl and the clearly visible peaks, we can estimate that the detection limit was around tenth of pg/µl.

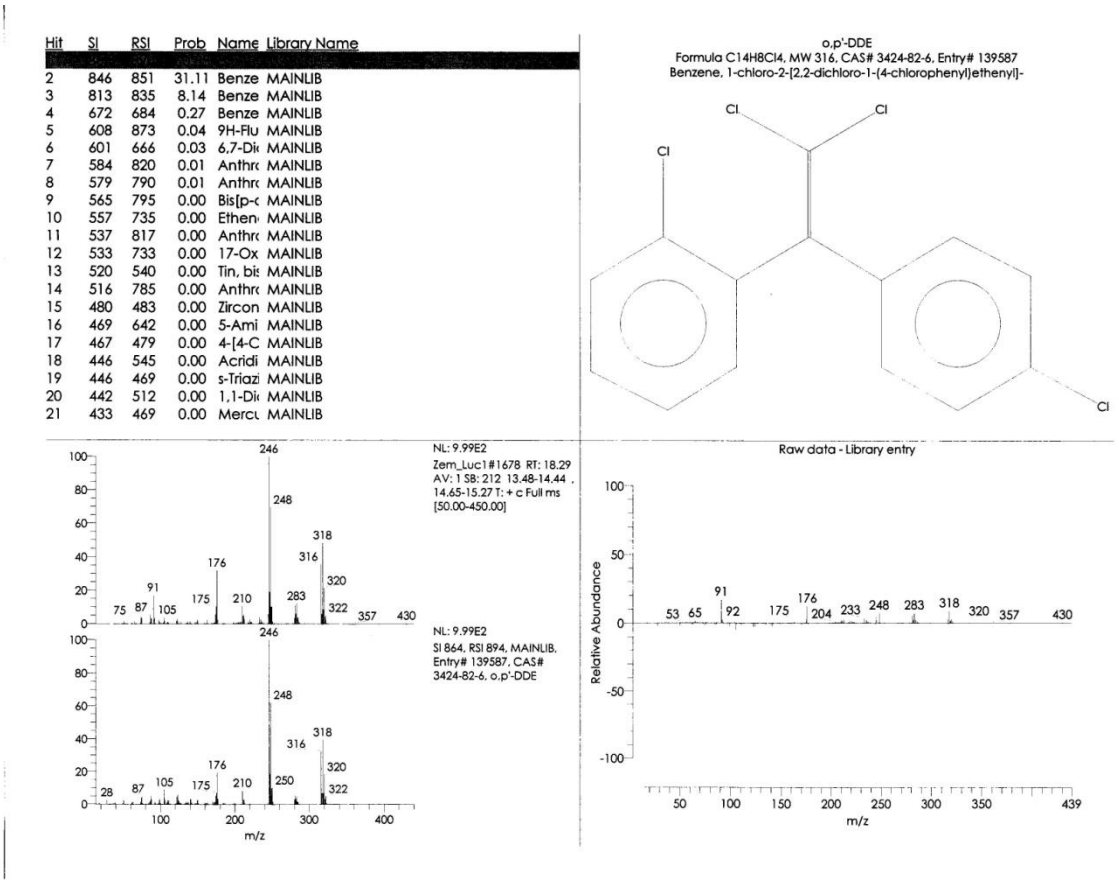
The soil sample was extracted with a mixture of acetone and hexane (2:3) in ultrasonic bath, the filtrated solvent was evaporated by rotary evaporator, then by nitrogen flow to dryness, 1 ml of toluene was added, and 1 µl of the final solution was injected to the GC-ECD instruments.

As a result from the soil sample analyzed by GC-ECD we expected a detection of PCBs usually found in the environment, especially PCB 153 and PCB 138, in some detectable concentration. Our expectation was confirmed- PCB 138 was detected in a concentration of 191.67 ng per gram of dry residue. When recalculated to µg per kilogram, that value is 191.67 µg/kg of the soil. This value is higher compare with the literature, where the concentration of PCB 138 in soil is in average 3.848 µg/kg (Danielovič, et al., 2003). On the other hand, this value is much lower than the value of PCBs measured in the soil nearby PCB-containing buildings, where the average concentration is in the range of 3.3 to 34 mg/kg (Herrick, et al., 2007). Therefore we can conclude that the soil near Spolana Neratovice chemical plant is still contaminated by PCB 138, even though that PCB production in former Czechoslovakia was banned in 1984. The GC-ECD chromatogram showed that there are other chlorinated compounds in the soil sample but we were unable to determine them exactly due to missing the appropriate standards.

The sample was also analyzed using GC-MS. This method is less sensitive in full scan mode for halogen containing compounds than GC-ECD and it is not possible to determine the exact positions of the chlorine atoms without standards. Also GC-ECD is also not able to distinguish between isomers (congeners) without standards. Anyway, three PCBs congeners with 6 chloro- substituents were detected. In the sample they were also detected other compounds like polycyclic aromatic compounds, e.g. pyrene, triphenylene. From other chlorinated compounds hexachlorofulvene and also dichlorodiphenyldichloroethylene (DDE) resulting from DDT biodegradation were detected (Figure 16). It is very probable that DDE is one of the peaks found in GC-ECD chromatogram which was not exactly determined. Other unknown peak might be dichlorodiphenyltrichloroethane (DDT) which was also detected by

GC-MS. DDT was used as a pesticide and it was produced in Neratovice chemical plants in the past.

Figure 16: DDE detected in GC-MS



Exact determination of all compounds found in the soil sample from the area of Spolana Neratovice chemical plant and their concentration values would be very interesting, but it goes unfortunately beyond of this bachelor thesis framework.

Conclusion

According to the literature and performed analyses using GC-ECD and GC-MS techniques, both techniques are suitable and sensitive enough for PCB detection including congeners with *ortho*-substitution. The obtained results have showed slight contamination of the selected area of Neratovice chemical plant compared to the results published in various articles regarding chemical plants, which is in accordance with the toxicity results. Using GC-MS analysis other chlorine containing compounds have been found in the soil sample, e.g. DDT and DEE. Thus, the problems of PCBs and other bioaccumulating compounds found in nature should not be underestimated and further analysis and research how to degrade or fully destroy these dangerous compounds is necessary.

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Attachments

Attachment 1: List of PCB congeners introduced by Ballschmiter and Zell and accepted by IUPAC

No.	Subst. pattern	No.	Subst. pattern	No.	Subst. pattern	No.	Subst. pattern	No.	Subst. pattern
1	2	**43**	2,2',3,5	**85**	2,2‘,3,4,4‘	**127**	3,3‘,4,5,5‘	**169**	3,3‘,4,4‘,5,5‘
2	3	**44**	2,2',3,5'	**86**	2,2‘,3,4,5	**128**	2,2‘,3,3‘,4,4‘	**170**	2,2‘,3,3‘,4,4‘,5
3	4	**45**	2,2',3,6	**87**	2,2‘,3,4,5‘	**129**	2,2‘,3,3‘,4,5	**171**	2,2‘,3,3‘,4,4‘,6
4	2,2'	**46**	2,2',3,6'	**88**	2,2‘,3,4,6	**130**	2,2‘,3,3‘,4,5‘	**172**	2,2‘,3,3‘,4,5,5‘
5	2,3	**47**	2,2',4,4'	**89**	2,2‘,3,4,6‘	**131**	2,2‘,3,3‘,4,6	**173**	2,2‘,3,3‘,4,5,6
6	2,3'	**48**	2,2',4,5	**90**	2,2‘,3,4‘,5	**132**	2,2‘,3,3‘,4,6‘	**174**	2,2‘,3,3‘,4,5,6‘
7	2,4	**49**	2,2',4,6	**91**	2,2‘,3,4‘,6	**133**	2,2‘,3,3‘,5,5‘	**175**	2,2‘,3,3‘,4,5‘,6
8	2,4'	**50**	2,2',4,6	**92**	2,2‘,3,5,5‘	**134**	2,2‘,3,3‘,5,6	**176**	2,2‘,3,3‘,4,6,6‘
9	2,5	**51**	2,2',4,6'	**93**	2,2‘,3,5,6	**135**	2,2‘,3,3‘,5,6‘	**177**	2,2‘,3,3‘,4‘,5,6
10	2,6	**52**	2,2',5,5'	**94**	2,2‘,3,5,6‘	**136**	2,2‘,3,3‘,6,6‘	**178**	2,2‘,3,3‘,5,5‘,6
11	3,3'	**53**	2,2',5,6'	**95**	2,2‘,3,5‘,6	**137**	2,2‘,3,4,4‘,5	**179**	2,2‘,3,3‘,5,6,6‘
12	3,4	**54**	2,2‘,6,6‘	**96**	2,2‘,3,6,6‘	**138**	2,2‘,3,4,4‘,5‘	**180**	2,2‘,3,4,4‘,5,5‘
13	3,4'	**55**	2,3,3‘,4	**97**	2,2‘,3‘,4,5	**139**	2,2‘,3,4,4‘,6	**181**	2,2‘,3,4,4‘,5,6
14	3,5	**56**	2,3,3‘,4‘	**98**	2,2‘,3‘,4,6	**140**	2,2‘,3,4,4‘,6‘	**182**	2,2‘,3,4,4‘,5,6‘
15	4,4'	**57**	2,3,3‘,5	**99**	2,2‘,4,4‘,5	**141**	2,2‘,3,4,5,5‘	**183**	2,2‘,3,4,4‘,5‘,6
16	2,2',3	**58**	2,3,3‘,5‘	**100**	2,2‘,4,4‘,6	**142**	2,2‘,3,4,5,6	**184**	2,2‘,3,4,4‘,6,6‘
17	2,2',4	**59**	2,3,3‘,6	**101**	2,2‘,4,5,5‘	**143**	2,2‘,3,4,5,6‘	**185**	2,2‘,3,4,5,5‘,6
18	2,2,5'	**60**	2,3,4,4‘	**102**	2,2‘,4,5,6‘	**144**	2,2‘,3,4,5‘,6	**186**	2,2‘,3,4,5,6,6‘
19	2,2',6	**61**	2,3,4,5	**103**	2,2‘,4,5‘,6	**145**	2,2‘,3,4,6,6‘	**187**	2,2‘,3,4‘,5,5‘,6
20	2,3,3'	**62**	2,3,4,6	**104**	2,2‘,4,6,6‘	**146**	2,2‘,3,4‘,5,5‘	**188**	2,2‘,3,4‘,5,6,6‘
21	2,3,4	**63**	2,3,4‘,5	**105**	2,3,3‘,4,4‘	**147**	2,2‘,3,4‘,5,6	**189**	2,3,3‘,4,4‘,5,5‘
22	2,3,4'	**64**	2,3,4‘,6	**106**	2,3,3‘,4,5	**148**	2,2‘,3,4‘,5,6‘	**190**	2,3,3‘,4,4‘,5,6
23	2,3,5	**65**	2,3,5,6	**107**	2,3,3‘,4‘,5	**149**	2,2‘,3,4‘,5‘,6	**191**	2,3,3‘,4,4‘,5‘,6
24	2,3,6	**66**	2,3‘,4,4‘	**108**	2,3,3‘,4,5‘	**150**	2,2‘,3,4‘,6,6‘	**192**	2,3,3‘,4,5,5‘,6
25	2,3',4	**67**	2,3‘,4,5	**109**	2,3,3‘,4,6	**151**	2,2‘,3,5,5‘,6	**193**	2,3,3‘,4‘,5,5‘,6
26	2,3',5	**68**	2,3‘,4,5‘	**110**	2,3,3‘,4‘,6	**152**	2,2‘,3,5,6,6‘	**194**	2,2‘,3,3‘,4,4‘,5,5‘
27	2,3',6	**69**	2,3‘,4,6	**111**	2,3,3‘,5,5‘	**153**	2,2‘,4,4‘,5,5‘	**195**	2,2‘,3,3‘,4,4‘,5,6
28	2,4,4'	**70**	2,3‘,4‘,5	**112**	2,3,3‘,5,6	**154**	2,2‘,4,4‘,5,6‘	**196**	2,2‘,3,3‘,4,4‘,5‘,6
29	2,4,5	**71**	2,3‘,4‘,6	**113**	2,3,3‘,5‘,6	**155**	2,2‘,4,4‘,6,6‘	**197**	2,2‘,3,3‘,4,4‘,6,6‘
30	2,4,6	**72**	2,3‘,5,5‘	**114**	2,3,4,4‘,5	**156**	2,3,3‘,4,4‘,5	**198**	2,2‘,3,3‘,4,5,5‘,6
31	2,4',5	**73**	2,3‘,5‘,6	**115**	2,3,4,4‘,6	**157**	2,3,3‘,4,4‘,5‘	**199**	2,2‘,3,3‘,4,5,6,6‘
32	2,4',6	**74**	2,4,4‘,5	**116**	2,3,4,5,6	**158**	2,3,3‘,4,4‘,6	**200**	2,2‘,3,3‘,4,5‘,6,6‘
33	2',3,4	**75**	2,4,4‘,6	**117**	2,3,4‘,5,6	**159**	2,3,3‘,4,5,5‘	**201**	2,2‘,3,3‘,4‘,5,5‘,6
34	2',3,5	**76**	2‘,3,4,5	**118**	2,3‘,4,4‘,5	**160**	2,3,3‘,4,5,6	**202**	2,2‘,3,3‘,5,5‘,6,6‘
35	3,3',4	**77**	3,3‘,4,4‘	**119**	2,3‘,4,4‘6	**161**	2,3,3‘,4,5‘,6	**203**	2,2‘,3,4,4‘,5,5‘,6
36	3,3',5	**78**	3,3‘4,5	**120**	2,3‘,4,5,5‘	**162**	2,3,3‘,4‘,5,5‘	**204**	2,2‘,3,4,4‘,5,6,6‘
37	3,4,4'	**79**	3,3‘,4,5‘	**121**	2,3‘,4,5‘,6	**163**	2,3,3‘,4‘,5,6	**205**	2,3,3‘,4,4‘,5,5‘,6
38	3,4,5	**80**	3,3‘,5,5‘	**122**	2‘,3,3‘,4,5	**164**	2,3,3‘,4‘,5‘,6	**206**	2,2‘,3,3‘,4,4‘,5,5‘
39	3,4',5	**81**	3,4,4‘,5	**123**	2‘,3,4,4‘,5	**165**	2,3,3‘,5,5‘,6	**207**	2,2‘,3,3‘,4,4‘,5,6,6‘
40	2,2',3,3'	**82**	2,2‘,3,3‘4	**124**	2‘,3,4,5,5‘	**166**	2,3,4,4‘,5,6	**208**	2,2‘,3,3‘,4,5,5‘,6,6‘
41	2,2',3,4	**83**	2,2‘,3,3‘,5	**125**	2‘,3,4,5,6‘	**167**	2,3‘,4,4‘,5,5‘	**209**	2,2‘,3,3‘,4,4‘,5,5‘,6,6‘
42	2,2',3,4'	**84**	2,2‘,3,3‘,6	**126**	3,3‘,4,4‘,5	**168**	2,3‘,4,4‘,5‘,6		