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Short Communication

Hormonal activities of new brominated flame retardants

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ABSTRACT

After the phase-out of two commercial mixtures of brominated flame retardants, an increasing number of alternative flame retardants have been introduced in commercial applications. None of them, however, has been thoroughly tested for its hormonal activity. We used two yeast reporter-gene assays to determine the potential of eleven compounds to interfere with estrogenic and androgenic pathways. Our data demonstrate the ability of 2,4,6-tribromophenol to lower the transcriptional activity of human estrogen and androgen receptors. A nominal IC_{50} value of 14.1 μM for anti-estrogenic and 3.9 μM for anti-androgenic activity was obtained using the luciferase reporter. An IC_{50} value of 9.2 μM was calculated for the anti-estrogenic activity measured by the β-galactosidase assay. Of the tested chemicals, this study highlights the endocrine disrupting effects of 2,4,6-tribromophenol whose occurrence in the environment should be monitored.

1. Introduction

Over the past decade, many researchers have focused on a wide range of anthropogenic chemicals that can be found in the environment. One group of these chemicals is called brominated flame retardants (BFRs). BFRs are added to many products widely used in households, such as TVs, electronics, polyurethane foam, and textiles (de Wit, 2002). Most BFRs are of anthropogenic origin; however, bromophenols were found to be biosynthesized in marine biota (Sim et al., 2009). BFRs reduce the likelihood of ignition of materials and/or decrease the rate of combustion leading to greater consumer safety. A great many articles have reported their presence in indoor dust and the environment (Wilford et al., 2004; Law et al., 2006; Allen et al., 2007; Sjodin et al., 2008), their accumulation in human tissues (Sjodin et al., 2003; Hites, 2004; Christiansson et al., 2008) and their toxicity (Fowles et al., 1994; Hallgren et al., 2001; Zhou et al., 2001, 2002; Stoker et al., 2005). Consequently, two commercial mixtures (penta-BDE and octa-BDE) have been voluntary withdrawn or banned from use. These two mixtures are also listed in the list of persistent organic pollutants (POPs; Stockholm Convention, 2009a,b: Decision SC-4/14 and Decision SC-4/18). After the phase-out of these two mixtures, many new and alternative brominated flame retardants has been used, but none of them have been thoroughly tested for its impact on the human endocrine system. This represents a serious problem, because previous studies of BFRs demonstrated the ability of previously used BFRs to damage the human endocrine system (Meerts et al., 2001; Samuelsen et al., 2001; Zhou et al., 2001). Some BFRs are suspected to behave in the same way as endocrine disrupters (EDs), due to their potential ability to bind to the Ah or other receptors and act *via* hormone-like mechanisms. The most frequently used flame retardants are structurally similar to polychlorinated biphenyls (PCBs) that have been documented to exibit endocrine disrupting activity (Hooper and McDonald, 2000; Meerts et al., 2001; Svobodova et al., 2009).

Consequently, this paper is focused on new brominated flame retardants and their ability to bind and activate estrogen and androgen receptors. We tested 1,2-bis(2,4,6-tribromophenoxy)ethane (TBPE), bis(2-ethylhexyl)tetrabromophthalate (BTBP), tetrabromophthalic anhydride (TBPA), 2,3,4,5,6-pentabromoethylbenzene (PBEB), 2,3,4,5,6-pentabromotoluene (PBT), 2,4,6-tribromophenol (TBP), hexabromobenzene (HBB), 2-allyloxy-1,3,5-tribromobenzene (ATBB), pentabromobenzyl acrylate (PBBA), tetrabromobisphenol A bis(dibromopropyl ether) (TBBPA-DBPE) and decabromobiphenyl (DBB). To date, only a few articles have reported the fate and behavior of these new BFRs in household dust and the environment as a whole (Gauthier et al., 2007; Verreault et al., 2007; Stapleton et al., 2008) and their toxicity (Brown et al., 2004). However, our knowledge of these substances remains very limited.

2. Materials and methods

2.1. Chemicals

Bis(2-ethylhexyl)tetrabromophthalate (TBPH, 99.5%, GC/MS), decabromobiphenyl (DBB, N/A), 1,2-bis(2,4,6-tribromophenoxy)

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ethane (BTBPE, N/A) and pentabromotoluene (PBT, N/A) were obtained from AccuStandard (USA). 2,3,4,5,6-Pentabromoethylbenzene (PBEB, 98%) and tetrabromobisphenol A bis(dibromopropyl ether) (TBBPA-BDPE, 99%) were obtained from Dr. Ehrenstorfer (Germany). The following chemicals were obtained from Sigma–Aldrich (Germany): tetrabromophthalic anhydride (TBPA, 98%), 2,4,6-tribromophenol (TBP, 99%), hexabromobenzene (HBB, 98%), pentabromobenzyl acrylate (PBBA, 98%), 2-allyloxy-1,3,5-tribromobenzene (ATBB, 98%), testosterone (Tes, ⩾99%) and 17β-estradiol (E2, 98%). All the tested chemicals were dissolved and diluted in a mixture of dimethyl sulfoxide (DMSO, ⩾99.9%, Sigma–Aldrich Germany) and water (30/70; v/v). The concentrations of the stock solutions of the chemicals, which were practically the highest concentrations attained (tested by HPLC; data not shown), are summarized in Table 1. Chlorophenol Red-β-D-galactopyranoside (CPRG, ⩾90%, for HPLC) was obtained from Fluka (Germany). Synthetic D-luciferin was obtained from Sigma–Aldrich (Germany).

2.2. β-Galactosidase assay

The estrogen-like activity of the tested chemicals was measured using a recombinant strain of *Saccharomyces cerevisiae*, producing β-galactosidase in response to estrogen exposure; this strain was cultivated and the tests were performed in 96-well plates according to Routledge and Sumpter (1996). The absorbance of cultures cultivated with the samples was measured at 540 nm and 620 nm and recalculated using the formula:

$$\text{Correlated value} = A_{540nm}\text{chemical} - (A_{620nm}\text{chemical} - A_{620nm}\text{blank})$$

The value represents the test response corrected for possible sample toxicity.

The test was performed in that the stock solutions were diluted 10-fold with the media containing the respective yeast strain. The anti-estrogen activities were assessed by testing the ability of the chemicals to decrease the yeast response evoked by 3.7×10^{-3} μM of E2 in the reaction mixture. 3% DMSO served as a blank for all the tests and the cultures were also cultivated without addition of DMSO to evaluate its possible toxic effect. All the measurements were performed in triplicate.

2.3. Bioluminescent estrogen and androgen screens

The bioluminescent yeast strains *S. cerevisiae* BMAEREluc/ERα, *S. cerevisiae* BMAEREluc/AR, and *S. cerevisiae* BMA64luc described by Leskinen et al. (2005) were used. Tests were carried out as described previously (Svobodova et al., 2009) using a Lumino-M90a luminometer (ZD Dolní Újezd, Czech Republic) and 60 s integration time for the luminescence detection. All the measurements were performed in triplicate. The induction of the sensors by the sample was calculated as the fold induction (FI) and the corrected fold induction ($FI_{corrected}$) together with the sample toxicity according to Leskinen et al. (2005).

The test was performed in that the stock solutions were diluted 11 times with the media containing the respective yeast strain. The anti-hormonal activities were evaluated by testing the abilities of the chemicals to diminish the yeast response evoked by 30×10^{-3} μM of E2 and 29×10^{-3} μM of Tes in all cases. 3% DMSO served as a blank for all the tests and the cultures were also cultivated without the addition of DMSO to evaluate its possible toxic effect.

2.4. Data analysis

To assess the estrogenicity and androgenicity of the compounds, the corrected values of the absorbance or luminescence versus the common logarithm of the chemical compound concentrations were

Table 1
Tested compounds and their toxicity towards the yeast sensor strains. The values represent means of three independent cultivations. The relative standard deviations of the values reached maximally 12%. The values describing toxicity for the β-galactosidase test represent a ratio of optical densities with and without the addition of BFRs. The correction factor for the luminescent test represents a luminescence ratio of the control strain BMA64luc without and with the addition of BFRs.

Name	CAS number	Abbreviation	Stock solutions concentrations (μM in 30% DMSO	Stock solutions concentrations (μg/ml in 30% DMSO)	Toxicity in β-galactosidase test $A620_{sample}/A620_{blank}$	Toxicity in β-galactosidase anti-estrogen test $A620_{sample}/A620_{blank}$	Toxicity in luminescent test correction factor	Toxicity in luminescent anti-estrogen test correction factor	Toxicity in luminescent. anti-androgen test correction factor
1,2-Bis(2,4,6-tribromophenoxy) ethane	37853-59-1	BTBPE	145.4	100	0.642	0.674	0.952	0.985	1.315
Bis(2-ethylhexyl) tetrabromophthalate	26040-51-7	TBPH	28.3	20	0.782	1.105	1.712	1.023	1.356
Tetrabromophthalic anhydride	632-79-1	TBPA	1078.2	500	0.660	0.710	1.013	1.005	1.025
2,3,4,5,6-Pentabromoethylbenzene	85-22-3	PBEB	39.9	20	0.754	1.154	1.019	0.920	1.252
2,3,4,5,6-Pentabromotoluene	87-83-2	PBT	41.1	20	0.700	1.022	1.062	0.983	1.084
2,4,6-Tribromophenol	118-79-6	TBP	604.6	200	0.237	0.252	1.020	0.368	0.461
Hexabromobenzene	87-82-1	HBB	36.3	20	1.019	1.313	0.950	0.972	1.262
2-Allyloxy-1,3,5-tribromobenzene	3278-89-5	ATBB	53.9	20	1.150	1.593	1.026	0.962	1.234
Pentabromobenzyl acrylate	59447-55-1	PBBA	35.9	20	1.078	1.036	0.890	0.787	0.795
Decabromobiphenyl	13654-09-6	DBB	21.2	20	0.698	1.110	1.075	0.963	1.122
Tetrabromobisphenol A bis (dibromopropyl ether)	21850-44-2	TBBPA-BDPE	21.2	20	1.003	1.084	1.005	0.970	1.077

plotted for each chemical. For hormonally active compounds, this relation should follow a sigmoid curve. In anti-hormonal assays, the measured yeast response to standard hormones (E2, Tes) in the presence of the tested BFRs was expressed as a percentage of the test response to positive controls consisting of E2 (3.7×10^{-3} μM for β-galactosidase test and 30×10^{-3} μM for luminescent test) and Tes (29×10^{-3} μM) for the estrogen and androgen assays, respectively.

Nominal IC_{50} values were calculated from two dose–response independent experiments (both performed in triplicate) as the nominal chemical concentrations at which 50% of the test response inhibition is reached. All the data including determination of IC_{50} were processed with OriginPro 8.5 (OriginLab, USA) software where the test responses were approximated by sigmoid curves and the upper asymptotes were set at 100%.

For the β-galactosidase assay, the toxicity of compounds towards the sensor yeast strains was evaluated by comparing the absorbance of the test and control cultures measured at 620 nm. For the luminescence assays, the correction factor (Cf) was used to assess the chemical toxicity towards the control strain *S. cerevisiae* BMA64luc, as described by Leskinen et al. (2005).

In the estrogen and androgen screens, the control cultures consisted of yeast cultures grown in the presence of 3% DMSO. In anti-hormonal assays, the control cultures additionally contained 3.7×10^{-3} μM of E2 in the β-galactosidase assay and 30×10^{-3} μM of E2 or 29×10^{-3} μM of testosterone in the luciferase reporter assay.

3. Results

3.1. Toxicity of compounds towards sensor strains

The toxicity of compounds towards the β-galactosidase producing strain of *S. cerevisiae* was assessed by comparing the absorbance of the test and control cultures measured at 620 nm, with or without added BFRs, respectively (Table 1). All the chemicals, except TBP, yielded absorbance ratios for the highest tested chemical concentrations in the range 0.66–1.15, indicating only partial yeast growth inhibition in some cases. Due to dilution with the medium, the yeast cultures were exposed to 10 times lower BFR concentrations than the stock solution concentration given in Table 1. 60.5 μM of TBP in the reaction mixture (containing 3% DMSO) exhibited a culture absorbance ratio of 0.237, signifying a toxic effect of the chemical on the sensor strain. A similar result (absorbance ratio of 0.252) was obtained when 60.5 μM of TBP in the mixture was mixed with E2 in the anti-estrogen screen. 30.3 μM in the mixture of TBP together with E2 already had no effect on the yeast growth (absorbance ratio for 30.3 μM of TBP = 1.087). DMSO (3%) did not show any significant toxic effect toward the strains on the basis of comparison of the absorbances at 620 nm (*t*-test, $P < 0.05$).

In the luminescent estrogen and androgen screening assay, no toxic effect of the tested compounds on the yeast growth was observed. This evaluation was performed by comparing the luminescences of the control luminescent strain BMA64luc with or without sample additions (Leskinen et al., 2005). On the other hand, when mixed with standard hormones, 60.5 μM of TBP displayed an stimulation effect on the luminescence of the control strain exhibiting a Cf values of 0.37 and 0.46 for the anti-estrogen and anti-androgen screening assays, respectively. These values did not fit the assay parameters according to Leskinen et al. (2005), which should be within the interval 0.5–2.0. For lower TBP concentrations, the Cf factor equaled 0.77-0.53 and was within the range acceptable for test evaluation. DMSO (3%) did not exhibit any significant toxic effect toward the strains compared to the luminescent values of the control strain (*t*-test, $P < 0.05$).

3.2. Estrogenic and androgenic activities of compounds

The estrogen screen based on the production of β-galactosidase in response to estrogens showed standard performance with E2, responding positively through the concentration range 3.7×10^{-5} to 3.7×10^{-3} μM. However, none of the tested BFRs was able to trigger β-galactosidase production in the yeast cultures, even at the highest tested concentrations in the reaction mixtures, equal to 5.4 μM for ATBB, 3.6 μM for HBB, 3.6 μM for PBBA, 2.1 μM for TBBPA-BDBE, 4.0 μM for PBEB, 4.1 μM for PBT, 2.1 μM for DBB, 2.8 μM for TBPH, 14.5 μM for BTBPE, 60.5 μM for for TBP and 107.8 μM for TBPA (Fig. 1). Similarly, no estrogenic activity was observed using a luminiscence assay for screening the compounds. The test responses to the highest tested chemical concentrations are shown in Fig. 1. The same BFRs were tested for their androgenic activities with the sensor strain *S. cerevisiae* BMAERELuc/AR (Fig. 2). The results indicated that the chemicals possess no significant androgen-like activity. The statistical significances of all the above results were tested using single sample *t*-test ($P < 0.05$).

3.3. Anti-hormonal activity of the compounds

In the assay based on the β-galactosidase production, TBPH, TBPA, PBEB, PBT, HBB, ATBB, PBBA, DBB, and TBBPA-BDPE did not affect the test response towards 3.7×10^{-3} μM of E2. On the other hand, 14.5 μM of BTBPE was able to lower β-galactosidase production by about 12.2% and the test response was inhibited up

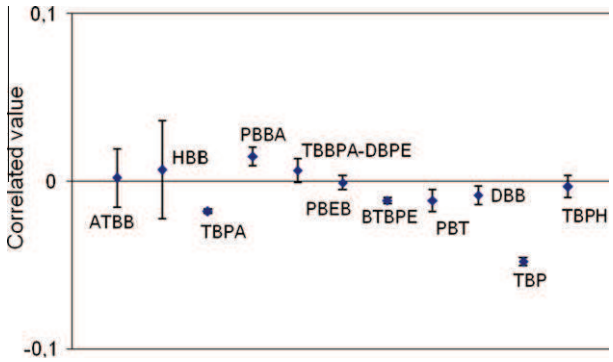

Fig. 1. Estrogenic activity of the tested compounds at the highest tested concentrations: 5.4 μM ATBB, 3.6 μM HBB, 3.6 μM PBBA, 2.1 μM TBBPA-BDBE, 4.0 μM PBEB, 4.1 μM PBT, 2.1 μM DBB, 2.8 μM TBPH, 14.5 μM BTBPE, 60.5 μM TBP, and 107.8 μM TBPA. The correlated value represents the test response corrected for the sample toxicity and the value 0 means no activity. The errors bars represent standard deviations.

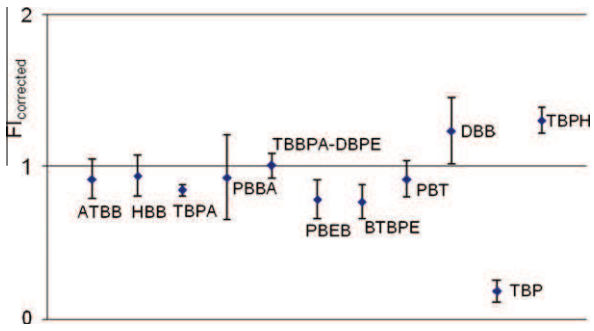

Fig. 2. Androgenic activity of the tested compounds at the highest tested concentrations: 4.9 μM ATBB, 3.3 μM HBB, 3.2 μM PBBA, 1.9 μM TBBPA-BDBE, 3.6 μM PBEB, 3.7 μM PBT, 1.9 μM DBB, 2.5 μM TBPH, 13.1 μM BTBPE, 54.4 μM TBP, and 98 μM TBPA. $FI_{corrected}$ represents the induction of the sensors corrected by the sample toxicity and the value 1 means no activity. The errors bars represent standard deviations.

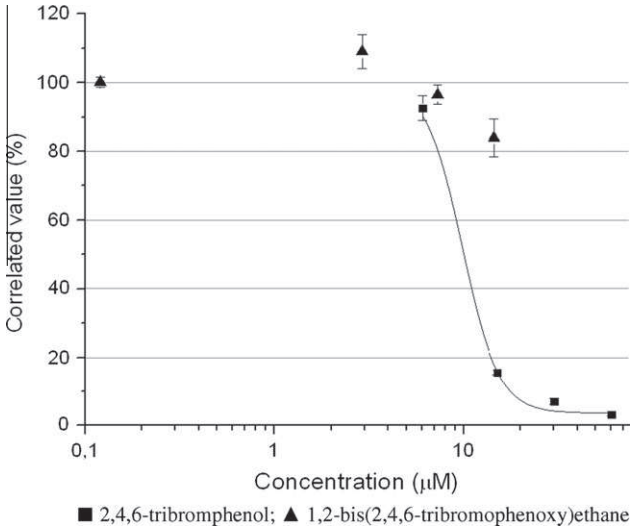

Fig. 3. Anti-estrogenic activity of TBP and BTBPE determined by the β-galactosidase assay. The correlated value means the test response corrected for the sample toxicity. 100% represents no inhibition of the E2 response.

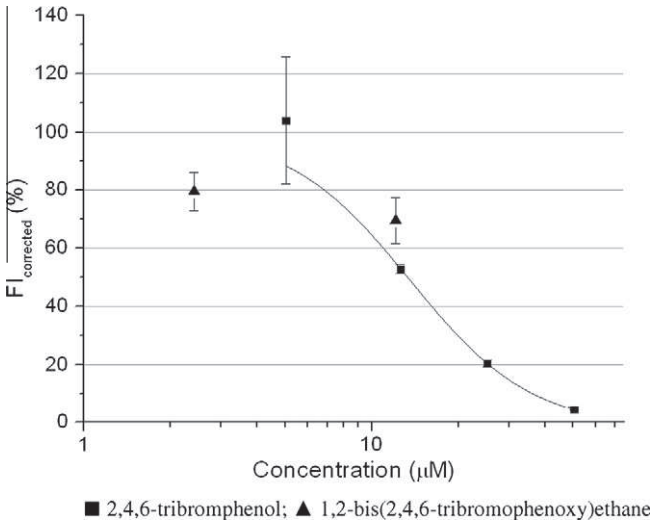

Fig. 4. Anti-estrogenic activity of TBP and BTBPE determined by bioluminescent screen. FIcorrected represents the induction of the sensors corrected for the sample toxicity. 100% represents no inhibition of the E2 response.

to 96.5% by TBP (Fig. 3). 60.5 μM of TBP inhibited the test response almost completely. However, this TBP concentration exhibited a certain toxic effect toward the yeast cultures. The second lower concentration (30.3 μM) also had a significant antagonist effect ($P < 0.05$). The nominal IC_{50} value calculated for TBP was 9.2 μM.

Anti-estrogenic activity of TBP was also observed using the luminescent assay. The nominal IC_{50} value was 14.1 μM in this case. BTBPE at a concentration of 12.1 μM also inhibited the yeast luminescence by 31% (Fig. 4). The antagonist effects were significant ($P < 0.05$) for TBP and BTBPE.

Of all tested chemicals only TBP exhibited substantial anti-androgenic activity (Fig. 5). TBP was able to lower the yeast bioluminescence caused by 29×10^{-3} μM of testosterone through the concentration range 0.2–50.4 μM. The nominal IC_{50} value was 3.9 μM. PBBA also inhibited slightly the bioluminescence by 25.5% at a concentration of 3.0 μM (data not shown). However, due to the low solubility of PBBA in the assay mixture the IC_{50} value was not determined. The antagonist effects were significant for TBP and PBBA ($P < 0.05$).

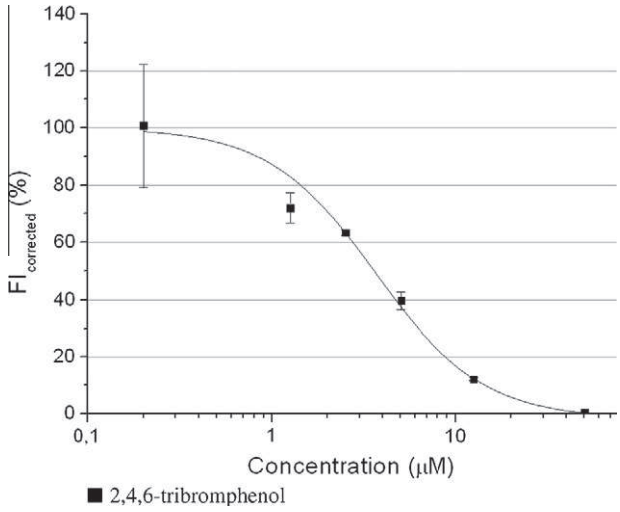

Fig. 5. Anti-androgenic activity of TBP determined by bioluminescent screen. $FI_{corrected}$ 1 represents 100% response of testosterone. $FI_{corrected}$ represents the induction of the sensors corrected for the sample toxicity. 100% represents no inhibition of the Tes response.

4. Discussion

The obtained results yielded the information that 2,4,6-tribromophenol exhibited anti-estrogenic and anti-androgenic hormonal activities. Cytotoxicity could be disregarded for the tested concentrations, where only the highest measured concentration of TBP exhibited a certain toxic effect towards the yeast. However, the second highest concentration already had no toxic effect but still inhibited the hormonal receptor at a significant level ($P < 0.05$).

The interaction of TBP with the androgen receptor has been described previously. Larsson et al. (2006) predicted that this compound can interact with an active site at an androgen receptor and interaction energies have been calculated reaching 27–28 kcal/mol. The molecule of TBP is too small to be able to interact properly with both polar ends of the active site of the receptor. TBP was found to interact mainly with the Asn and Thr groups, but was not able to reach across the active site to Arg88 (Larsson et al., 2006). These findings are in agreement with our results where TBP did not show any activation of AR in this study. It can be concluded that this chemical substance had an antagonist effect on AR. Another study measured the anti-androgenic activity of TBP using the CALUX bioassay. An IC_{50} value of >15 μM was found; however, this value was calculated using only one sample and the cytotoxicity could not be neglected (Hamers et al., 2006). This study yielded the value IC_{50} = 3.9 μM which is lower than the value of 15.0 μM obtained by Hamers et al. (2006). The difference between the results may be due to different *in vitro* assays, where Hamers et al. used the CALUX bioassay with human osteoblasts.

Olsen et al. (2002) measured the binding affinities of 2,4,6-tribromophenol for the ER. TBP exhibited a relative binding affinity of approximately 0.0004 compared with 17β-estradiol. The authors found that TBP was unable to affect ER-dependent cell proliferation and showed no sign of an antagonist effect (Olsen et al., 2002). Another study described the ER antagonistic response and yielded the value IC_{50} = 8.3 μM (Hamers et al., 2006). These results are similar to the IC_{50} value found in our study, reaching values of 14.1 μM and 9.2 μM for the bioluminescent and the β-galactosidase assay, respectively.

TBP has been detected in rivers and the seawater. Sim et al. (2009) suggested that e.g. *via* biosynthesis some marine algae (Flodin and Whitfield, 1999) can significantly contribute to the formation of

TBP in the seawater. However, the presence of the compound in a fresh water environment is probably predominantly derived from anthropogenic origin.

In contrast to TBP, hormonal activities of compounds BTBPE and PBBA remains unknown. This article is the first one that describes and demonstrates the anti-estrogenic activity of BTBPE and anti-androgenic activity of PBBA. These substances are not sufficiently soluble in our tests to discover the concentration dependence of the antagonistic effect in order to calculate their IC_{50} values. The results emphasize the need for application of other more complex tests (with human tissues for example) to investigate the endocrine activities of these substances.

5. Conclusions

The antagonist effects of TBP on ER and AR were demonstrated using two reporter-gene screens. The results indicate possible binding of this chemical to an active site of the receptors and they exhibited a potential ability to almost completely inhibit the activity of the receptors. This fact documents the substantial environmental potency of TBP, which should be monitored in the environment. Several articles have described concentrations of TBP in the human environment and even in human tissues (Thomsen et al., 2001; Reineke et al., 2006; Kawashiro et al., 2008; Sim et al., 2009; Takigami et al., 2009). Due to these facts, this compound could be classified as a new environmental endocrine disruptor.

BTBPE and PBBA should be tested with other bioassays to demonstrate their anti-hormonal activities and calculated IC_{50}. This will lead to better understanding of the endocrine potential of these compounds.

Acknowledgments

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Mechanistic Study of 17α-Ethinylestradiol Biodegradation by *Pleurotus ostreatus*: Tracking of Extracelullar and Intracelullar Degradation Mechanisms

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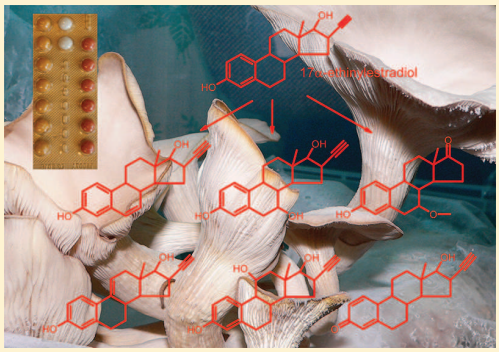
ABSTRACT: The white rot fungus *Pleurotus ostreatus* is able to completely remove the synthetic hormone 17α-ethinylestradiol (EE2, 200 μg in 20 mL) from a liquid complex or mineral medium in 3 or 14 days, respectively. Its efficiency has also been documented in the removal of estrogenic activity that correlated with the EE2 degradation. A set of in vitro experiments using various cellular and enzyme fractions has been performed and the results showed that EE2 was degraded by isolated laccase (about 90% within 24 h). The degradation was also tested with concentrated extracellular liquid where degradation reached 50% mainly due to the laccase activity; however, after a supplementation with H_2O_2 and Mn^{2+}, residual manganese-dependent peroxidase activities (40 times lower than Lac) raised the degradation to 100%. Moreover, the intracellular fraction and also laccase-like activity associated with fungal mycelium were found to be efficient in the degradation too. Isolated microsomal proteins appeared to also be involved in the process. The degradation was completely suppressed in the presence of cytochrome P-450 inhibitors, piperonylbutoxide and carbon monoxide, indicating a role of this monooxygenase in the degradation process. Attention was also paid to monitoring of changes in the estrogenic activity during these particular in vitro experiments when mainly degradations related to ligninolytic enzymes were found to decrease the estrogenic activity with EE2 removal proportionally. Several novel metabolites of EE2 were detected using different chromatographic method with mass spectrometric techniques (LC-MS, GC-MS) including also [^{13}C]-labeled substrates. The results document the involvement of various different simultaneous mechanisms in the EE2 degradation by *P. ostreatus* by both the ligninolytic system and the eukaryotic machinery of cytochromes P-450.



INTRODUCTION

Synthetic estrogen 17α-ethinylestradiol (EE2) is widely used in oral contraceptive pills.[1] Trace concentrations (from ng to μg per liter) of EE2 and its conjugates, have been detected in wastewater treatment plants,[2,3] their effluents,[4−6] rivers,[7−9] groundwaters,[10,11] and also in a drinking water reservoir.[7] To reduce ecological as well as human health risks, development of novel approaches for removing EE2 from the environment are required.

A possible approach in estrogens elimination consists of physicochemical treatment (advanced oxidation processes, granulated activated carbon adsorption, etc.).[6,12,13] Some of the methods are very effective, but their high cost for maintenance or safety requirements limit their widespread use in water purification. Moreover, the tendency of EE2 to adsorb onto sludge, together with low biodegradation efficiency of wastewater treatment processes, leads to its accumulation. The sludge is used as fertilizers (biosolids) representing another source of the pollution.[1,6,10] In general, controlling and optimization of biodegradation processes require the knowledge of the occurring degradation mechanisms, including characterization of the transformation products and their biological effects.

Ligninolytic (white rot) fungi have been studied from this point of view as well. They possess a unique degrading enzyme apparatus and it has been documented that they are able to biodegrade a wide range of organic pollutants. This is mainly due to their ability to excrete low-substrate specific extracellular enzymes: lignin peroxidase (LiP), manganese-dependent peroxidases (MnP), manganese-independent (versatile) peroxidases (MiP), and laccase (Lac).[14] However, other enzymes could also be involved in the biodegradation of EE2, e.g. 17β-hydroxysteroid dehydrogenase in reduction of steroids.[15] It is

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also known that the degradation of organic pollutants could also be attributed to fungal microsomal enzymes, e.g. cytochrome P-450 monooxygenase.[16,17]

Degradation of EE2 by whole cultures of white rot fungi has been described.[18,19] Several studies investigated applications of white rot fungal Lac[20−23] and MnP[24] for EE2 removal. A certain role of fungal intracellular enzymes, such as cytochrome P-450, in the biodegradation of some natural steroids as well as other xenobiotics, has been also suggested.[25−27] Nonetheless, only a limited number of articles deal with the characterization and localization of enzyme machineries responsible for EE2 degradation or elucidation of intracellular vs extracellular enzymes participation in the EE2 decomposition. Several authors also monitored estrogenic changes during the fungal EE2 biodegradation.[18,24,28] Estrogenic activities generally decreased as the degradation process progressed. However, in some cases, the results from the bioassays showed a residual or temporally increased estrogenic activity over the course of the degradation, suggesting the production of degradation intermediates with estrogenic activity. Thus, monitoring of changes in the endocrine-disrupting activity could be another useful tool for understanding and evaluating the biodegradation efficiency. The identification of EE2 metabolites can provide an additional insight into the degradation mechanism and can help to elucidate the enzyme participation in the transformation processes and moreover to assess the toxicological risks related to the degradation processes of EE2. However, limited information is available about biotransformation products of EE2 and only several abiotic EE2 degradation products resulting from chemical-catalytic processes have been reported to date,[29−31] together with a few publications focused on EE2 metabolic studies by bacteria,[32−34] algae,[35] and by filamentous fungi.[36]

This study was performed in order to investigate particular enzyme systems and cell fractions of *Pleurotus ostreatus* with respect to their abilities to degrade EE2. The fungal strain was selected according to our previous study.[18] The result showed *P. ostreatus* was a very efficient and promising microorganism for biodegradation of EE2. The degradation performance of the whole cell cultures was examined in two different model liquid nutrient media and further, in vitro experiments were designed and carried out to elucidate the participation of intracellular and extracellular enzymes in the EE2 degradation process. Removal of estrogenic activity was monitored and several EE2 metabolites were identified. To our knowledge, this is the first article describing ligninolytic fungal metabolites of EE2 and characterizing the involvement of particular enzymes in the degradation, including ligninolytic enzymes, intracellular fraction, and possible involvement of cytochrome P-450.

MATERIALS AND METHODS

Chemicals. EE2 (98%) was used as a substrate for degradation experiments and was purchased from Aldrich, Germany. [^{13}C]-EE2 (99%) was obtained from Cambridge Isotope Laboratories, Inc. Other chemicals (pure or of higher purity) used as standards were supplied by Fluka, Aldrich, or Merck (Germany). All the solvents of trace analysis quality or gradient grade were purchased from Chromservis (Czech Republic).

Organisms and Cultivation. The ligninolytic fungal strain *Pleurotus ostreatus* 3004 CCBAS 278 was obtained from the Culture Collection of Basidiomycetes of the Academy of Sciences, Prague. Two different liquid media were used in this study: N-limited liquid mineral medium (LNMM) containing 2.4 mM diammonium tartrate,[37] and complex malt extract glucose medium (MEG), containing 5 and 10 g·L^{-1} of malt extract broth (Oxoid, Basingstoke, UK) and glucose, respectively. Inocula were prepared in 250-mL Erlenmeyer flasks (ErF) containing 20 mL of MEG or LNMM starting from 2 mycelial plugs (0.7 mm Ø). Cultures grown for 7 days at 28 °C were homogenized by Ultraturrax-T25 (IKA-Labortechnik, Staufen, Germany), and 5% aliquots of the mycelial suspension were used to inoculate static cultures. The static 20- or 50-mL cultures (MEG or LNMM) were incubated in 250- or 500-mL ErF at 28 °C, respectively.

Preparation of Mycelium and Extracellular Liquids for in Vitro Biodegradation Experiments. Mycelium and extracellular liquids of 4-day-old cultures (MEG) cultivated in 500-mL ErF were separated using a filter paper. The mycelium was washed with distilled water and homogenized using Ultraturrax-T25 (IKA-Labortechnik, Germany) in 300 mM ammonium acetate buffer (pH 4.5). Extracellular liquids were concentrated using 10 kDa cutoff membrane centrifugal units (Millipore, USA; conditions: 4000*g*, 10 min, 4 °C).

Microsomal Fraction Purification for in Vitro Biodegradation Experiments. The microsomal fraction from *P. ostreatus* was isolated from 12-day-old static cultures (500-mL ErF, 120 flasks) in MEG medium.[26] The mycelium was filtered on nylon cloth, washed with cold phosphate buffer (100 mM, pH 7.2), and disrupted in a Virtis 45 blender at 375 Hz in the same buffer supplemented with glycerol (200 g·L^{-1}) and bovine serum albumin (1.5 g·L^{-1}). The supernatant was centrifuged twice (10 000*g*, 15 min, and then 100 000*g*, 90 min). The pellets from the second centrifugation were then suspended in the same buffer, centrifuged (100 000*g*, 90 min), suspended again, and stored in phosphate buffer (50 mM, pH 7.2) containing glycerol (300 g·L^{-1}), ethylenediaminetetraacetic acid (EDTA, 0.1 mM), and glutathione (GSH, 0.1 mM). The obtained pellets were referred to as the microsomal fraction and used for in vitro biodegradation experiments. The total protein content in the microsomal fraction was determined by the dye-binding method using bovine serum albumine as the standard.[38]

Laccase Semipurification for in Vitro Biodegradation Experiments. The culture liquid from 12-day-old *P. ostreatus* cultures (500-mL ErF, 60 flasks) was separated from the mycelium by filtration on a paper filter and concentrated 60-fold using a 10 kDa cutoff membrane (Whatman, USA) at 4 °C. The retentate was filtered using cellulose membrane 0.22 μm, Whatman, USA, and further applied to a desalting Hi-Prep column (26/10; GE Healthcare, USA) equilibrated in 20 mM sodium phosphate buffer (pH 6.0) at a flow rate of 3 mL·min^{-1}. The protein fraction was applied to an anion exchange column packed with Mono Q 5/50 GL (Amersham Pharmacia, USA) and Lac was eluted by NaCl gradient (0 mM, 13 min; 0 to 1000 mM, 38 min; and 1000 mM, 10 min) in sodium phosphate buffer (monitoring of 215, 280, and 405 nm). Fractions with Lac activity were pooled and again desalted using a Hi-Prep column, stored in 10% glycerol (v/v), and used for in vitro incubations.

Enzyme Assays and Determination of Estrogenic Activity. The LiP activity was assayed with veratryl alcohol as the substrate[37] and MnP activity was measured as described by de Jong et al.[39] The MiP activity was calculated from the peroxidase activity of MnP assay detected in the absence of Mn^{2+} ions. Lac activity was determined by oxidation of 5 mM

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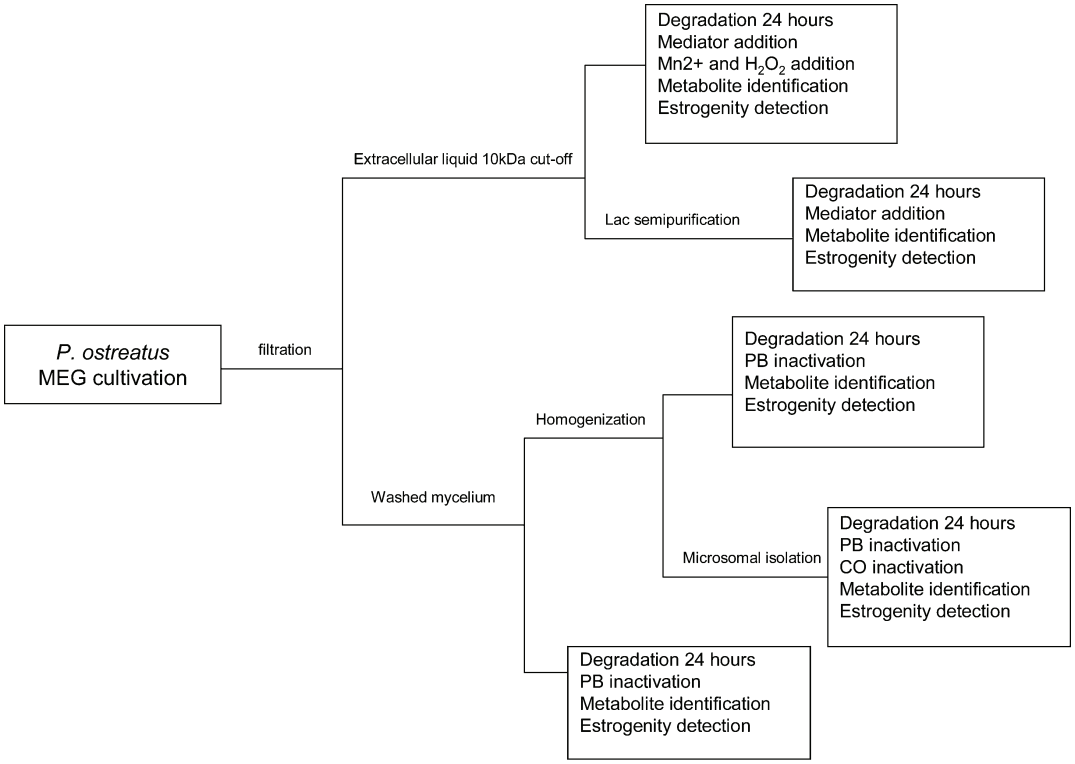

Figure 1. Schematic overview of the experimental in vitro setup.

2,2-azinobis-3-ethylbenzo-thiazoline-6-sulfonic acid.[40] One unit of enzyme produced 1 μmol of the reaction product per min under the assay conditions at room temperature. Mycelium associated laccase-like enzyme activity was determined according to Svobodová et al.[41] To determine the estrogenic activity, the ethyl acetate extracts were evaporated and redissolved in 30% dimethylsulfoxide (DMSO, in water, v/v) and the activity was further assessed using a recombinant yeast screen according to Routledge and Sumpter.[42]

Degradation Experiments. *P. ostreatus* was tested to degrade EE2 in vivo in MEG and LNNM media. A set of in vitro degradation experiments was performed using *P. ostreatus* fractions after cultivation in MEG. An overview of the experimental in vitro approach is described in Figure 1. In all of the degradation experiments we quantified residual EE2 concentration, determined estrogenic activity, and tried to detect degradation products by GC-MS and LC-MS techniques.

The samples for in vivo biodegradation experiments were prepared in two types of liquid media (20 mL in 250-mL EmF) in three parallels and spiked immediately after the inoculation with 100 μL of EE2 (2 mg·mL^{-1} in DMSO, final concentration 10 μg·mL^{-1} of EE2) and harvested after 3, 7, and 14 days of cultivation. Biotic controls (BC) were spiked with only 100 μL of DMSO. The abiotic heat-killed controls (HKC) were performed with mycelium grown for one week and killed in an autoclave.

All the in vitro experiments were performed in triplicate in 4-mL reaction tubes closed with Teflon-lined screw caps. Incubations were performed at 28 °C for 24 h on a rotary shaker (80 rpm) in the dark. Buffer controls were prepared using a spiking solution, 60 mM acetate buffer (pH 4.5), and only water. HKC were prepared by adding heat-denatured (60 min, 100 °C) fractions.

Mycelial degradation samples were performed in 2-mL reaction mixtures containing 70 mg of wet nonhomogenized or homogenized mycelium, 60 mM acetate buffer (pH 4.5), and 10 μg·mL^{-1} of EE2. The ABTS oxidizing enzyme activity was determined as 81.9 ± 7.2 U·g^{-1} of dry mycelium and the amount of dry mycelium in the samples was about 21 mg. Controls were performed by addition of piperonylbutoxide (PB) to the reaction mixtures to suppress the intracellular P-450 activity.

Degradation experiments with concentrated extracellular liquid were performed in 2-mL reaction mixtures containing 60 mM acetate buffer (pH 4.5), 10 μg·mL^{-1} of EE2, and about 10-fold concentrated extracellular liquid containing Lac (activity 1.0 U), MnP (activity 0.02 U). MiP activity was not detected. Another set of samples was incubated with addition of 200 mM $MnSO_4$ and 10 mM H_2O_2 to the reaction mixtures.

Lac degradation samples were prepared in 1-mL reaction mixtures containing 1.0 U of Lac, 60 mM acetate buffer (pH 4.5), 10 μg·mL^{-1} of EE2, and 1 mM hydroxybenzoic acid (HBA) for the mediated samples. The other ligninolytic activities were not detectable in this case.

Degradation samples with microsomal fraction in 1-mL reaction mixtures contained 0.5 mg of microsomal proteins resuspended in 100 mM potassium phosphate buffer (pH 7.2), 4 μg·mL^{-1} EE2, 1 mM EDTA, 0.2 mM NADPH, 1 mM phenylmethanesulfonylfluoride, 1 mM dithiothreitol, and 2% DMSO (v/v). Controls were inactivated by addition of PB or by carbon monoxide (CO) flux into the reaction mixtures.

EE2 Quantification. In vitro degradation samples were acidified by addition of 20 μL of 1 M HCl and extracted by an aliquot of ethyl acetate (EtOAc, 2 mL; 5 times). The extracts

were then dried with sodium sulfate and concentrated to dryness using a nitrogen stream. Samples were diluted in 1 mL of acetonitrile and analyzed using an HPLC-UV system (Alliance, Waters 2695 Separations Module, Waters, Milford, MA) equipped with a diode-array detector (Waters 2996). An isocratic program was used with 70% acetonitrile in water (v/v). EE2 was separated on a XBridge C18 column (250 mm × 4 mm, particle diameter 3.5 μm), provided by Waters, with a flow rate of 0.8 mL·min^{-1} at 35 °C. The detection limit for EE2 was 0.44 mg·L^{-1}. The extraction recovery of EE2 was better than 85%.

In vivo degradation samples were acidified and measured directly after addition of 0.5 mL of acetonitrile and centrifugation (5 min, 10 000 rpm) using the same HPLC method as described above. The mycelium parts were also extracted with EtOAc to confirm that the removal of EE2 was not caused by adsorption onto the fungal biomass.[43]

Metabolites Identification: GC/MS. EtOAc extracts (0.3 mL of each) were derivatized at 50 °C for 60 min using 0.5 mL of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA): Trimethylsilane (TMS) (99:1, v/v) and 0.3 mL of pyridine. The samples were then evaporated to dryness using a nitrogen stream, and 0.5 mL of EtOAc was added. Analyses for metabolite identification were performed with a Varian 450-GC (USA) instrument equipped with a Combi-Pal injector (CTC Analytics, Sweden) and a Varian 240 MS ion trap detector employing an electron impact ion source. One μL of the sample solution was injected into the GC-MS systems. The analyses were performed with a 30 m long × 0.25 mm I.D., 0.25 mm film thickness, DB-5MS column (Agilent, USA). The GC oven temperature program started from 60 °C (hold 1 min), then heated up to 100 °C at 25 °C·min^{-1}, then to 135 °C at 1 °C·min^{-1}, and finally to 240 °C at 15 °C·min^{-1} and held isothermally for 30 min. Helium (99.999%) was used as the carrier gas, with a constant flow rate of 1 mL·min^{-1}. The injector was operated in the split/splitless mode, with a splitless time of 1 min. The injector temperature was 240 °C. The source, ion trap, and transfer line temperatures were 250, 220, and 280 °C, respectively. The mass spectra were recorded at 3 scans·s^{-1} under electron impact (EI) at 70 eV and mass range 50−450 amu. Methane (99.999%) was used for chemical ionization (CI). Structures were suggested by comparing the mass spectra with the data in the NIST 08 library and independently by interpreting the fragmentation pattern. MS/MS product ion mode experiments and CI were employed for better elucidation of the metabolite structures. [^{13}C]-labeled EE2 was also used as the substrate in parallel biodegradation experiments to confirm the relationship between EE2 and the found degradation products.

Metabolites Identification: LC/MS. The extracts were evaporated to dryness and redissolved in acetonitrile, and 15 μL aliquots were injected into HPLC-MS system. The apparatus consisted of an 1100 Series pump with am 1100 series autosampler (Hewlett-Packard, Germany) connected to a API 2000 triple-stage quadrupole mass spectrometer operating with an APPI interface (Applied Biosystems, USA) and Analyst 1.4 (Applied Biosystems) software. HPLC separation was performed on an Acqua C18 column (150 mm × 2.0 mm, particle diameter 3.5 μm, Phenomenex) at a flow rate of 0.3 mL min^{-1} using water (A) and acetonitrile (B) in a linear gradient (0 min, 20% B; 15 min, 80% B; and 16 min, 20% B). The analytes were detected at negative ion mode. The nebulizer gas, heater gas, curtain gas flow, and collision gas were set at 420.0, 435.5, 20.1, and 20.0 kPa, respectively. The lamp protecting gas was set at 1 mL·min^{-1} at APPI mode. The source block was maintained at 200 °C and ion spray voltage was set at −5500 V. Nitrogen was used as curtain and collision gas. For metabolite identification, full scan analysis in the mass range of 80−800 amu was performed and product ion scan analyses were used to investigate EE2 metabolites more in detail.

Statistical Tests. The results represent means of the respective parallel analyses. Their significant differences were tested using t test ($P < 0.05$). The uncertainties of the means are displayed as their respective standard deviations.

RESULTS AND DISCUSSION

In vivo EE2 Degradation and Determination of the Estrogenic Activities. As previously reported in MEG cultures, EE2 and other EDCs are able to influence enzyme activity profiles.[18] In the current study, a significant decrease in the Lac activity of about 50% was observed in the MEG cultures in the presence of EE2 (Table 1; t test, $P < 0.05$), similarly to the previous study.[18] In addition, strong suppression of MnP activity was recorded after EE2 addition to the LNMM cultures (about 10 times) and the enzyme activity peak maximum shifted from day 14 to day 9.

Table 1. Detected Maximum Ligninolytic Enzyme Activities (U·L^{-1}) in Extracellular Liquid Measured during Biodegradation Experiments (Supplemented with EE2) and in Cultivations without EE2 (Control)[a]

	Lac		MiP		MnP	
	max	day	max	day	max	day
control MEG	75 (4)*	2−4	8 (3)	2−4	32 (9)	4
EE2MEG	45 (13)*	2−4	8 (2)	2	20 (2)	4
control LNMM	4 (2)	9	ND		37 (7)*	14
EE2 LNMM	6 (2)	9	ND		3 (1)*	9

[a]The values are the means of three replicates, and the values in brackets are the standard deviations. ND = not detected. The values labeled with an asterisk were found to be significantly different (t test, $P < 0.05$).

P. ostreatus is able to degrade EE2 in liquid media (Figure 2). The data indicate that the degradation in LNMM medium is slower in comparison with that in MEG medium. While the EE2 concentration in the MEG medium decreased below the respective limit of detection (0.2 μg·mL^{-1}) within the first 3 days of cultivation, a significant degradation in the LNMM medium was not observed in this period (t test, $P < 0.05$). A decrease to about 20% of the initial EE2 concentration occurred after the first 7 days of cultivation in LNMM medium and the total EE2 removal was recorded after 14 days. The media represent very different cultivation conditions (mineral N-limited and complex) and despite the differences between the results, the data document ability of *P. ostreatus* to efficiently degrade EE2 under both of the conditions. To make metabolite identification more reliable, [^{13}C]-labeled EE2 was degraded in the same way and finally, the degradation course of the labeled EE2 in MEG medium was the same as for the unlabeled substrate (data not shown). The profile of estrogenic activities during the biodegradation did not perfectly correlate with the EE2 concentration decrease (Figure 2, correlation coefficient for MEG 0.73, for LNMM 0.92). The level of estrogenic activity in the 3 d and 7 d samples is higher than the observed EE2 levels in both cases. The delay in the decrease in

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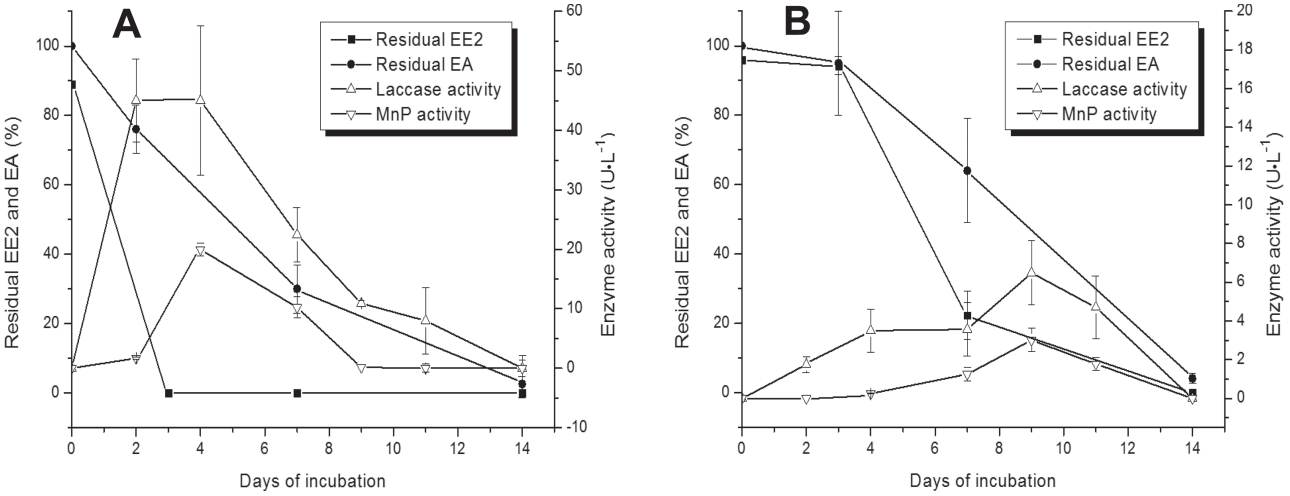

Figure 2. In vivo EE2 degradation by *P. ostreatus* in MEG (A) and LNMM (B) media (20 mL in 250 mL EmF): residual concentration of EE2, estrogenic activity (EA), and the course of ligninolytic enzyme activities during biodegradation. The left axis (100%) represents 10 μg·mL^{-1} of EE2 and original estrogenic activity of the samples (maximal response of the test).

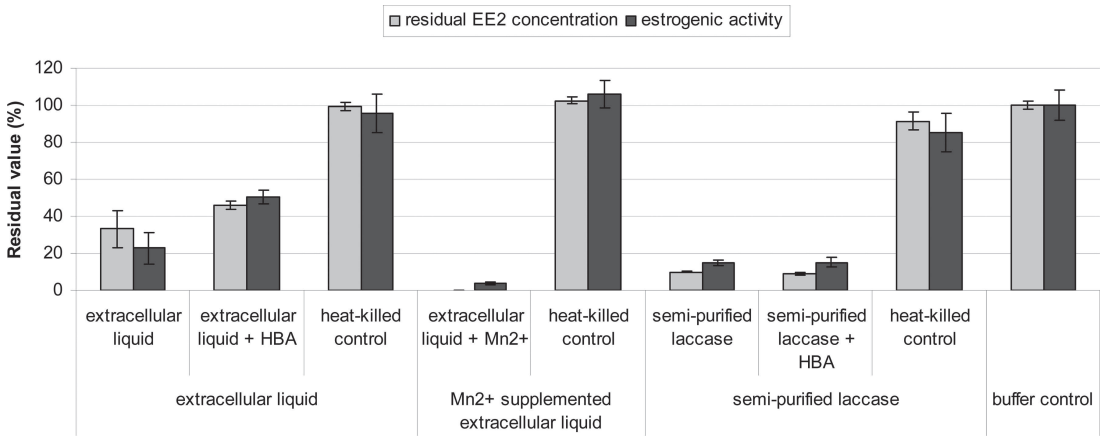

Figure 3. Extracellular in vitro EE2 degradation (24 h) and estrogenic activity determination in samples with extracellular liquid, extracellular liquid supplemented with Mn^{2+} and hydrogen peroxide and semipurified Lac. HBA = hydroxybenzoic acid. 100% represents 10 μg·mL^{-1} of EE2 and its corresponding estrogenic activity.

the estrogenic activities suggests a temporal formation of metabolites possessing estrogenic activity during the initial phases of biodegradation by *P. ostreatus*.

Extracellular in Vitro EE2 Degradation. During the degradation experiments in MEG, high extracellular activities of Lac were detected (Table 1) whereas peroxidase activities were detected at lower level. Thus, a possible involvement of Lac in EE2 degradation was investigated employing (i) the concentrated extracellular liquid with mainly Lac activity (with detectable MnP and without detectable MiP activities, further, (ii) the semipurified Lac (without detectable MnP and MiP activities), and also (iii) the laccase-redox mediator hydroxybenzoic acid in the degradation experiments (Figure 3). These degradation experiments were performed without any EE2 induction during the fungal incubation. However, EE2 was not completely removed in any of these treatments within 24 h of incubation. To the contrary, total EE2 removal was observed in the samples supplemented with Mn^{2+} and hydrogen peroxide despite the substantially lower MnP activity in the extracellular liquid. The tested Lac and MnP activities in the extracellular liquid samples were 1.0 U and 0.025 U, respectively, and the activity in the semipurified Lac degradation samples was 1.0 U. The results indicate simultaneous biodegradation of EE2 by laccase and residual MnP. We also performed experiments where Lac was induced by the presence of EE2; however, the degradation efficiency with this EE2 induced Lac was about 50% lower (data not shown).

The estrogenic activity changes correspond to the decrease in the EE2 concentrations as displayed in Figure 3. This finding suggests that ligninolytic extracellular enzymes decompose EE2 in such a way that the formed metabolites lack activity or these intermediates are rapidly transformed further into other less active compounds. These results are in accordance with findings of other authors[24] who recorded almost complete removal of estrogenic activity simultaneously with EE2 degradation by MnP and Lac from *P. chrysosporium* and *T. versicolor*, respectively. Other authors have also reported biodegradation of EE2 with isolated enzymes from ligninolytic fungal strains such as Lac originated mainly from *Trametes versicolor*,[21,22] *P. ostreatus*, and *Pycnoporus coccineus*.[22]

Mycelium-Mediated and Intracellular in Vitro EE2 Degradation. The most effective (total) degradation of EE2

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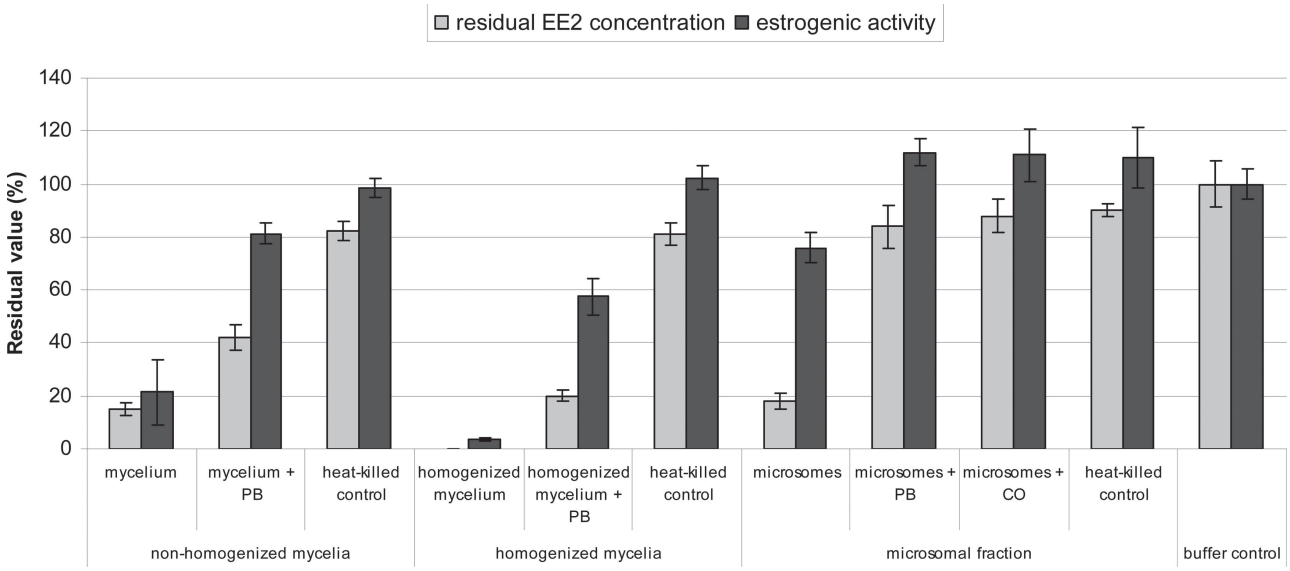
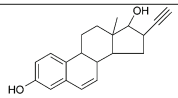
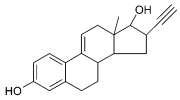
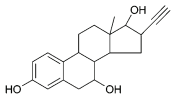
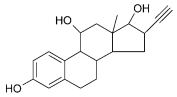
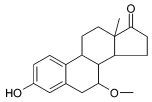
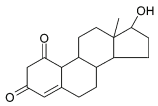


Figure 4. Intracellular and mycelial in vitro EE2 degradation (24 h) and estrogenic activity determination with washed mycelium, homogenized mycelium, and microsomal fraction. PB = piperonylburoxide; CO = carbon monoxide. 100% represents 10 $\mu g \cdot mL^{-1}$ of EE2 for mycelial samples and buffer control and its corresponding estrogenic activity. Microsomal degradation was performed with 4 $\mu g \cdot mL^{-1}$ of EE2.

Table 2. Characterization and Structural Assignment of the Detected EE2 Metabolites; the EI Spectra Belong to the Trimethylsilylated Derivatives of the Original Compounds

Metabolite assignment	Formula	Suggested metabolite structure	Metabolite related structure	Origin	Mw (CI)	EI characteristics Ions: m/z (% intensity)	Methods of characterization	Literature
1		**19-nor-17α-pregna-1,3,5 (10), 6-tetraen-20-yne-3,17β-diol**	**dehydrogenated EE2**	MEG cultivation	294	438 (21,3), 423 (12,9), 348 (1,3), 283 (16,0), 281 (14,0) 207 (21,2) 73 (99,9)	GC-MS, GC-MS^2, ^{13}C	
2		**19-nor-17α-pregna-1,3,5(10), 9 (11)-tetraen-20-yne-3,17 β-diol**	**dehydrogenated EE2**	MEG cultivation	294	438 (99,9), 423 (22,5), 333 (16,2), 283 (31,2), 229 (39,8), 207 (29,1), 73 (95,6)	GC-MS, GC-MS^2, LC-MS	
3		**19-nor-17α-pregna-1,3,5 (10)-trien-20-yne-3,7 (or 6)-17β-triol**	**hydroxylated EE2**	MEG cultivation; mycelial degradation	312	513 (10.2), 439 (9.2), 318 (56.8), 355 (19.5), 73 (99.9)	GC-MS, GC-MS^2, ^{13}C	36
4		**19-nor-17α-pregna-1,3,5 (10)-trien-20-yne-3,1 (or 2)-17β-triol**	**hydroxylated EE2**	MEG cultivation; mycelial degradation	312	513 (13.8), 355 (22.3), 217 (15.2), 73 (99.9)	GC-MS, GC-MS^2	35, 36
5		**6-methoxy-estrone**	**methoxylated E1**	LNMM cultivation	300	372 (99.9), 357 (10.2), 197 (38.1), 126 (26.3), 73 (20.8)	GC-MS	35
6		**19-nor-17α-pregna-4 (10)-en-17-hydroxy-1,3-dion**	**dioxo-E2**	Semipurified Lac	288	360 (89.3), 345 (97.7.), 285 (82.1), 270 (76.5)	GC-MS	

was recorded with homogenized mycelium where the laccase-like (ABTS oxidizing) activity was found to be associated with the mycelium (91.2 U per dry mycelium) and readily available microsomal intracellular fraction (Figure 4). In this experiment, we also detected the highest removal of estrogenic activity (residual 4%). After an application of piperonylbutoxide (PB), which is supposed to be an inhibitor of cytochrome P-450, we detected a drop in the degradation efficiency and a substantially higher residual estrogenic activity (58%) was recorded. A significantly lower degradation extent (t test, $P < 0.05$) was observed after the application of nonhomogenized mycelium, when 20% of the initially applied amount of EE2 was found. In this case, the degradation of EE2 seems to be performed mainly via extracellular enzyme activity; however, inhibition by PB again suggests the participation of intracellular cytochrome P-450. The difference between the degradation rates of

homogenized and nonhomogenized mycelium could be attributed to EE2 transport through the cell-wall and the membrane. The slight discrepancies between the recoveries in the heat-killed controls and their respective estrogenic activities could be caused by matrix effects to both of the assays. However, regarding this issue, heat-killed controls were realized in parallel for all of the conditions under study.

The ability of the intracellular microsomal fraction to degrade EE2 is documented in the third set of experiments where the isolated microsomes from *P. ostreatus* removed more than 80% of the applied amount of EE2. This reaction was completely inhibited by carbon monoxide or PB. It is worth noting that the results of the microsomal degradation were obtained independently of the addition of NADPH. However, Sono et al.[46] described unusual forms of P-450 containing an electron donor in one of their protein domains that can finally support the possible participation of P-450 cytochrome in the degradation as observed in our case. Recently published results showed that the degradation of various organic pollutants by white rot fungi could be attributed to the function of cytochrome P 450.[28,44−46]

Metabolite Identification. Table 2 lists the electron impact (EI) mass spectral characteristics, methods of detection, and the type of degradation experiment where the EE2 metabolites were determined. In all the degradation experiments, the intermediates and products appeared only at trace levels. All of the compounds were detected with GC-MS techniques after trimethylsilylation and the EI spectra were used for structural suggestions. Most of the EI spectra were explored using MS/MS (product ion scan) to clarify the fragmentation sequence and to increase the probability of structural assignment. Chemical ionization using methane was employed to find the molecular weight of unknown metabolites. In one case, the respective metabolite (no. 2) was also confirmed using the LC-APPI-MS technique of the non derivatized degradation mixture. The EE2 origin of metabolites 1 and 3 were confirmed in a parallel degradation experiment using [^{13}C]-labeled EE2. The structures of the detected degradation products are shown in Table 2. The results indicate that *P. ostreatus* was able to convert EE2 into several more polar metabolites.

The structures of the intermediates again suggest that the transformation of EE2 is mediated by several enzyme mechanisms. The dehydrogenated EE2 intermediates (1, 2) could be formed by the enzyme 17β-hydroxysteroid dehydrogenase or an enzyme with similar action. This enzyme is known to be involved in steroid degradation by *P. otreatus* culture.[47] In this work, metabolites 1 and 2 were proven to be formed during the cultivation with [^{13}C]-labeled EE2. Oxidation of the phenolic functional group in the case of metabolite 6 could be attributed to a laccase-catalyzed reaction. As copper-containing enzymes, Lac are able to efficiently oxidize phenols and anilines in the presence of oxygen.[14] The transformation of EE2 into E1 has already been observed by other authors after degradation using unspecified biofilm bacterial isolates from a river system and after degradation using *Sphingobacterium* sp.[33,34] Further methoxylation on the benzene ring of estrone (metabolite 5) could be caused by the consequent methylation of hydroxyl derivatives as it was observed for various other aromatic endocrine disrupting pollutants degraded by *P. ostreatus*.[50,51] This sequence probably requires hydroxylation by cytochrome P-450 and further action of methyl transferases. Hydroxylated EE2 (metabolites 3 and 4) were previously described and identified using NMR by Choudhary et al.[36] The authors identified several metabolites resulting from degradation of norethisterone by the nonligninolytic fungus *Cephalosporium aphidicola*, which transformed this steroid to EE2 and consequently to hydroxylated EE2. This fungus is also well-known for its ability to hydroxylate and modify other steroids and sesquiterpenoids.[48] Choudhary et al.[36] also found methylation of the hydroxyl EE2 metabolite by *Cephalosporum*. Moreover, they detected several other hydroxylated EE2 metabolites by zygomycete *Cunninghamella elegans*.[36] Likely, the majority of the fungal steroid hydroxylation reactions are catalyzed probably by cytochrome P450-dependent monooxygenases,[17] where all the stereogenic centers of steroids, are attacked quite nonspecifically and frequently in high yields.[49] In the case of *P. ostreatus*, P-450 may play an important role in the EE2 metabolism, but our results also documented that other enzyme apparatuses are involved. Thus the yields of simple hydroxylated products occur only at trace concentrations that do not allow any further isolation and application of spectrometric methods other than MS.

In summary, the presented data of the investigations suggest that a synergic action of several metabolic mechanisms is supporting an efficient EE2 degradation by *P. ostreatus*. Possible roles of the metabolic mechanisms could be attributed to intracellular degradation via microsomal enzymes, mycelium associated laccase-like activity, extracellular degradation via manganese-dependent peroxidase, and extracellular Lac activity. The possible contribution of the individual degradation activities is indicated and each degradation step was evaluated according to its ability to individually remove estrogenic activity. The data document that despite recalcitrance of EE2, this compound is efficiently degraded by ligninolytic system; additionally, also by the more general present eukaryotic machinery of cytochromes P-450. To our knowledge, this is the first article describing EE2 biodegradation products and the mechanisms possibly involved in the process by a ligninolytic fungus. This physiological group of basydiomycetes represents one of a few microorganisms representatives that are able to effectively decompose EE2.

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Notes

The authors declare no competing financial interest.

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Review

Ecotoxicity and biodegradability of new brominated flame retardants: A review

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ABSTRACT

Brominated flame retardants (BFRs) have been routinely used as additives in a number of consumer products for several decades in order to reduce the risk of fire accidents. Concerns about the massive use of these substances have increased due to their possible toxicity, endocrine disrupting properties and occurrence in almost all the environmental compartments, including humans and wildlife organisms. Several conventional BFRs (e.g. polybrominated diphenylethers (PBDE)) have been included in the list of Persistent Organic Pollutants and their use has been restricted because of their established toxicity and environmental persistence. Over the past few years, these compounds have been replaced with "new" BFRs (NBFRs). Despite the fact that NBFRs are different chemical molecules than traditional BFRs, most of physical–chemical properties (e.g. aromatic moiety, halogen substitution, lipophilic character) are common to both groups; therefore, their fate in the environment is potentially similar to the banned BFRs. Therefore, this article has been compiled to summarize the published scientific data regarding the biodegradability of the most widely used NBFRs, a key factor in their potential persistency in the environment, and their ecotoxicological effects on humans and test organisms. The data reviewed here document that the mechanisms through NBFRs exibit their ecotoxicity and the processes leading to their biotransformation in the environment are still poorly understood. Thus emphasis is placed on the need for further research in these areas is therefore emphasized, in order to avoid the massive use of further potentially harmful and recalcitrant substances of anthropogenic origin.

Contents

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0147-6513/

1. Introduction

To date, a large number of brominated flame retardants (BFRs) have been produced and introduced into the market. These substances are incorporated as additives in materials used in common household items such as TVs, electronics, polyurethane foams, textiles etc. (de Wit, 2002) in order to reduce the likelihood of ignition of these materials and/or decrease their rate of combustion; however, BFRs can increase the toxicity of fires through the release of byproducts such as brominated and chlorinated dioxins and furans during combustion. The incorporation of organohalogens can increase levels of carbon monoxide, irritant gases, and soot, the main factors resulting in fire deaths and injuries in fires. Therefore the anticipated improvement in consumer safety is highly disputable (DiGangi et al., 2010; Shaw et al., 2010). Some BFRs are not chemically bound to a polymeric matrix, leading to the risk of diffusion into the environment.

Three main groups of BFRs, namely polybrominated diphenylethers (PBDE), hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA) are considered "typical" or "conventional" flame retardants, since they were introduced into the market in the past. A number of scientific articles have dealt with the occurrence of these BFRs in the environment (Haneke, 2002a; Shaw and Kannan, 2009; Shaw et al., 2009; de Wit et al., 2010; Kefeni et al., 2011; Nyholm et al., 2013; Gorga et al., 2013), their biodegradation (Vrkoslavová et al., 2011; Eljarrat et al., 2011; Lal et al., 2010), and their accumulation in human and animal tissues (Hites, 2004; Inoue et al., 2006; Sjödin et al., 2003) and their toxicological properties (Birnbaum and Staskal, 2004; Viberg et al., 2004; Zhou et al., 2001) also including their non-specific aquatic baseline toxicity (Stieger et al., in press; Mayer and Reichenberg, 2006). The problem associated with their biodegradation in the environment is linked with the fact that they often content an aromatic moiety. Aromatic compounds are usually chemically resistant due to delocalization of energy, moreover, it has been suggested that the environmental persistence of conjugated compounds is due to the dense clouds of PI-electrons on both the sides of the ring structures, which greatly reduces their susceptibility to nucleophilic attack (Johnsen et al., 2005). The resistance is even greater when the structures are substituted with so-called "xenophores" e.g., halogens (Alexander, 1994). The problem is even more pronounced in the presence of bromine atoms, which are somewhat larger than other substituents and limit the potential for microbial intracellular transformation processes (Cajthaml and Svobodová, 2012).

The described negative effects of these BFRs (see below) prompted the production and commercialization of alternative organobromines, which are commonly used at the present time. It is the intention of this paper to review recent findings on the ecotoxicity and environmental risk associated to these newer and alternative compounds with aromatic structure, as well as their biodegradability.

2. Legislative regulations on BFRs in the European Union

Since a number of BFRs have been found to be persistent in the environment, legislative steps have been taken to discontinue their production. The European Union (EU) performed risk assessment for penta- and octabrominated diphenyl ethers according to the REACH Regulation (Registration, Evaluation and Authorization of Chemicals; Regulation (EC) No. 1907/2006). It was subsequently agreed to prohibit their sale and use in the EU. Since 2004, Directive 2003/11/EC had prohibited trading and use of PentaBDE and OctaBDE in products containing more than 0.1% of these substances by weight. Waste products containing more than 0.25% PentaBDE were classified as hazardous waste. With reference to the Directive on Waste Electrical and Electronic Equipment (2002/96/EC – also known as WEEE) and the Directive restricting the use of certain hazardous substances in electrical and electronic equipment (2002/95/EC – known as RoHS), an agreement was reached to put an end to the use of BFRs in electrical and electronic equipment by 1 July 2006. Directive 2002/95/EC was followed

Table 1
Properties of NBFR reviewed in this paper. The abbreviation system was adopted from Bergman et al. (2012).

Name	Abbreviation	CAS	Solubility	Melting point (°C)	Log K_{ow}	Log K_{oc}
1,2-Bis(2,4,6-tribromophenoxy)ethane	BTBPE	37853-59-1	0.72 μg/L[a]	350[a]	3.55[a]	6.1[d]
Decabromodiphenylethane	DBDPE	84852-53-9	2.1×10^{-7} g/L[b]	333–349[b]	11.1[b]	13.2[e]
Bis(2-ethylhexyl) tetrabromophtalate	BEH-TEBP	26040-51-7	< 0.05 μg/L[a]	−27[a]	10.2[a]	7.4[d]
Tetrabromophthalic anhydride	TEBP-Anh	632-79-1	241 mg/L[a]	298.8[a]	1.98[a]	3.43[g]
2,3,4,5,6-Pentabromoethylbenzene	PBEB	85-22-3	3.5×10^{-4} g/L[b]	138[b]	6.4[b]	4.86[g]
2,3,4,5,6-Pentabromotoluene	PBT	87-83-2	7.8×10^{-4} g/L[b]	280–289[b]	5.87[b]	4.57[g]
Hexabromobenzene	HBB	87-82-1	1.1×10^{-7} g/L[b]	326–327[b]	6.07[b]	4.56[g]
2-Allyloxy-1,3,5-tribromobenzene	TBP-AE	3278-89-5	0.02 g/L[b]	Not available	5.4[c]	4.08[g]
2,4,6-Tribromophenol	TBP	118-79-6	50 mg/L[a]	95.5[a]	3.27[a]	2.98[g]
Decabromobiphenyl	BB209	13654-09-6	$1.24 \times 10^{-10-11}$ g/L[h]	380–386[h]	12.63[f]	
Tetrabromobisphenol A bis(dibromopropyl ether)	TBBPA-BDBPE	21850-44-2	0.144 μg/L[a]	113.4[a]	10.3[e]	12.9[e]

[a] Data from Registered substances database, European Chemicals Agency (ECHA).
[b] Data from Covaci et al. (2011).
[c] Data from Bergman et al. (2012).
[d] Data from La Guardia et al. (2012).
[e] Data from Nyholm et al. (2013).
[f] Data from Doucette and Andren (1987).
[g] Data from Harju et al. (2009).
[h] Data from Hazardous Substances Data Bank (HSDB), U.S. National Library of Medicine.

by Commission Decision 2005/717/EC, which contains an exemption from the ban on the use of DecaBDE (Commission of the European Communities, 2003a, 2003b, 2003c, 2005a, 2005b, 2007, 2008).

In 2009, some BFRs were also added into the list of persistent organic pollutants (POPs) in the framework of the Stockholm Convention. These restrictions relate to commercial mixtures of PentaBDE and OctaBDE (decisions SC-4/18 and SC-4/14). Both these compounds were included in Annex A, which contains substances prohibited from use, production, import and export. The same list already included another flame retardant, hexabromobiphenyl (HexaBB), and other organic pollutants, such as polychlorinated biphenyls, hexachlorobenzene, toxaphene, etc. Recently, hexabromocyclododecane (HBCDD) has also been added to Annex A (2013, decision SC-6/13) with specific exemptions and for use in expanded and extruded polystyrene.

3. "New" brominated flame retardants

After the legislative ban on some of the most widely produced BFRs, many new and alternative flame retardants came into use on a wide scale. The acronym NBFRs is used in the scientific literature to define these novel brominated flame retardants. Nevertheless, there are a number of alternative terms which have been used to refer to BFRs other than PBDEs, HBCD and tetrabromobisphenol A (TBBPA) like "alternate", "new", "emerging", "current-use" or "non-PBDEs" (Covaci et al., 2011). These substances are either newly discovered or previously synthesized compounds that have become more extensively used in recent years.

The following subsections present a list of organobromides that are now used as flame retardants without any restrictions. This list does not include PBDE and HBCD due to the legislative restrictions on their use and also does not include TBBPA. The text is focused mainly on the toxicological and ecotoxicological properties of NBFRs, as well as on their potential to undergo biological degradation. In fact, although several review papers describe the occurrence and fate of these substances in the environment, the ecotoxicological and biodegradation aspects have not yet been reviewed. The physicochemical characteristics and structural formulas of the reviewed flame retardants are summarized in Table 1 and Fig. 1. Table 2 summarizes concentration levels detected in the environment that are further discussed also in the respective subsections. It is impossible to list all the brominated flame retardants due to the lack of available information regarding which BFRs are currently used. For instance, in Bergman et al. (2012) the reader can find a list of some other "new" and alternative BFRs that are out of the scope of this review.

3.1. 1,2-Bis(2,4,6-tribromophenoxy)ethane (BTBPE)

BTBPE is one of the alternative BFRs used as a replacement for OctaBDE and was launched on the market under the brand name FF 680 or firemaster 680. It is produced by the reaction of 1,2-dibromoethane with 2,4,6-tribromophenolate and is currently used as a flame retardant additive in acrylonitrile-butadiene styrene (ABS) resins, high impact polystyrene (HIPS) and electronic products such as computers, television sets and mobile phones (Tomy et al., 2007).

BTBPE has been detected in the ambient and indoor air (Hoh et al., 2005; Sjödin et al., 2001) and the compound was also found in lake sediments, in association with other BFRs. Its measured concentrations were lower than those of BDE209, but much higher than those of other PBDE congeners. Analysis of the core sediments from the Lake Michigan was related to the 1973–1985 period; the authors discovered a continuous and time-dependent increase in the BTBPE concentration, which corresponds to the long-term use of this compound in the USA (Hoh et al., 2005). BTBPE was also determined in household dust in various studies (Karlsson et al., 2007; Stapleton et al., 2008). Interestingly, BTBPE was detected in all the samples in the latter study, but still at levels considerably lower than PBDEs. In another study of household dust, BTBPE average concentrations were found to equal 120 ng/g in homes, 7.2 ng/g in offices and 7.7 ng/g in cars (Harrad et al., 2008).

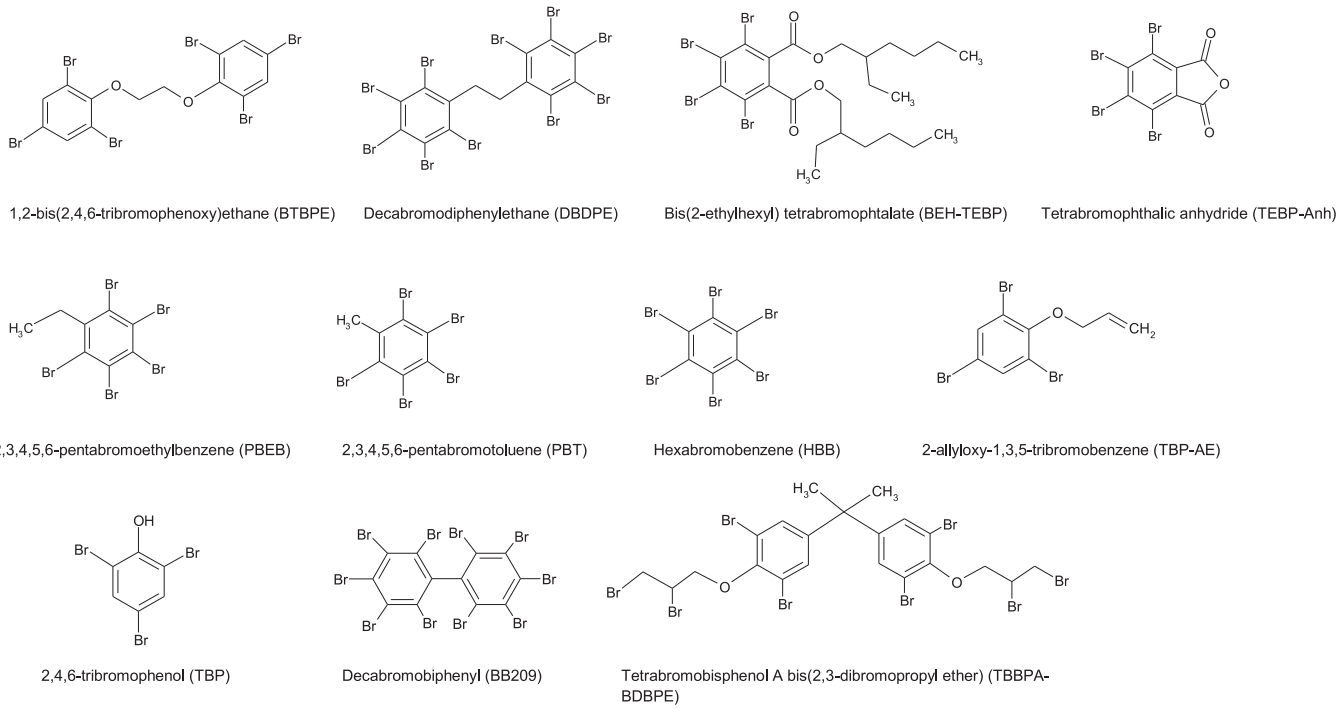

Fig. 1. Structural formulas of NBFR reviewed in this paper.

Table 2
Summary of the detected concentrations in the environment.

Sample	Compounds	Conc. range	Region	Reference
Ambient air	BTBPE	2.8–70 pg/m^3	USA	Hoh et al. (2005)
	BTBPE	3.83–67.4 pg/m^3	China	Shi et al. (2009)
	BTBPE	Average 0.4–1.8 pg/m^3	USA	Ma et al. (2013)
	BTBPE	ND – 0.06 pg/m^3	Greenland	Möller et al. (2011)
	BTBPE	Average 0.73 pg/m^3	China	Qiu et al. (2010)
	DBDPE	402–3578 pg/m^3	China	Shi et al. (2009)
	DBDPE	Average 1.2–5.2 pg/m^3	USA	Ma et al. (2013)
	DBDPE	Average 23 pg/m^3	China	Qiu et al. (2010)
	BEH-TEBP	Average 0.5–8 pg/m^3	USA	Ma et al. (2013)
	PBEB	29 and 520 pg/m^3	USA	Hoh et al. (2005)
	PBEB	Average 0.1–3 pg/m^3	USA	Ma et al. (2013)
	PBEB	ND – 0.01 pg/m^3	Canada	Gouteux et al. (2008)
	PBT	0.001–0.02 pg/m^3	Greenland	Möller et al. (2011)
	PBT	ND – 0.02 pg/m^3	Canada	Gouteux et al. (2008)
	HBB	Average 0.3–5.5 pg/m^3	USA	Ma et al. (2013)
	HBB	0.04–0.66 pg/m^3	Greenland	Möller et al. (2011)
	HBB	0.02–0.09 pg/m^3	Canada	Gouteux et al. (2008)
	HBB	0.3–6.5 pg/m^3	China	Qiu et al. (2010)
	TBBPA-BDBPE	131–1240 pg/m^3	China	Shi et al. (2009)
Indoor air	BTBPE	5.6–67 ng/m^3	Sweden	Sjödin et al. (2003)
	BTBPE	1.1–39 ng/m^3	Sweden	Pettersson-Julander et al. (2004)
	DBDPE	0.7 ng/m^3	Sweden	Kierkegaard et al. (2004)
	DBDPE	ND – 0.0229 ng/m^3	Sweden	Karlsson et al. (2007)
	DBDPE	0.01–1.2 ng/m^3	Sweden	Pettersson-Julander et al. (2004)
	BB209	1.6–14 ng/m^3	Sweden	Sjödin et al. (2001)
House dust	BTBPE	ND – 1900 ng/g	Birmingham, UK	Harrad et al. (2008)
	BTBPE	2.52–8.15 ng/g	Sweden	Karlsson et al. (2007)
	BTBPE	1.6–789 ng/g	USA	Stapleton et al. (2008)
	BTBPE	< 0.5–1741 ng/g	UK	Ali et al. (2011)
	BTBPE	< 0.5–1019 ng/g	Belgium	Ali et al. (2011)
	DBDPE	ND – 3400 ng/g	Birmingham, UK	Harrad et al. (2008)
	DBDPE	ND – 121 ng/g	Sweden	Karlsson et al. (2007)
	DBDPE	< 10–11,070 ng/g	USA	Stapleton et al. (2008)
	DBDPE	< 20–2467 ng/g	UK	Ali et al. (2011)
	DBDPE	55–2126 ng/g	Belgium	Ali et al. (2011)
	BEH-TEBP	1.5–10,630 ng/g	USA	Stapleton et al. (2008)
	BEH-TEBP	< 2–6175 ng/g	UK	Ali et al. (2011)
	BEH-TEBP	< 2–5004 ng/g	Belgium	Ali et al. (2011)
	TBP	16–620 ng/g	Japan	Takigami et al. (2009)
	TBP	< 20–2300 ng/g	Thailand	Muenhor et al. (2010)
	TBBPA-BDBPE	< 20–9961 ng/g	UK	Ali et al. (2011)
	TBBPA-BDBPE	< 20–2211 ng/g	Belgium	Ali et al. (2011)
Dust	BTBPE	14.6–232 ng/g	China	Shi et al. (2009)
	BTBPE	Average 1060 ng/g	Germany	Sawal et al. (2008)
	DBDPE	< 2.5–139 ng/g	China	Shi et al. (2009)
	DBDPE	Average 353 ng/g	Germany	Sawal et al. (2008)
	PBT	Average 0.97 ng/g	Germany	Sawal et al. (2008)
	HBB	Average 0.48 ng/g	Germany	Sawal et al. (2008)
	BB209	Average 37.9 ng/g	Germany	Sawal et al. (2008)
	TBBPA-DBDPE	Average 1300 ng/g	Germany	Sawal et al. (2008)
Sediment	BTBPE	Approximately 0.1–9 ng/g dry weight	USA	Hoh et al. (2005)
	BTBPE	0.27–21.9 ng/g	China	Shi et al. (2009)
	BTBPE	77–2000 ng/g	USA	La Guardia et al. (2012)
	BTBPE	Average 4554 ng/g	China	Wu et al. (2010)
	DBDPE	24 ng/g	Sweden	Kierkegaard et al. (2004)
	DBDPE	Average 1796 ng/g	China	Wu et al. (2010)
	DBDPE	4.8–24 ng/g	Spain	Guerra et al. (2010)
	DBDPE	38.8–364 ng/g	China	Shi et al. (2009)
	BEH-TEBP	2000–19200 ng/g	USA	La Guardia et al. (2012)
	PBEB	Average 132 ng/g	China	Wu et al. (2010)
	PBEB	ND – 9.6 ng/g	Spain	Guerra et al. (2010)
	PBT	< 1–25 ng/g	Germany	Schwarzbauer et al. (2001)
	HBB	Average 8672 ng/g	China	Wu et al. (2010)
	HBB	ND – 2.4 ng/g	Spain	Guerra et al. (2010)
	TBP	0.56–12.3 ng/g	Korea	Sim et al. (2009)
	TBBPA-BDBPE	< 1.5–2300 ng/g	China	Shi et al. (2009)
Soil	BTBPE	0.02–0.11 ng/g	China	Shi et al. (2009)
	DBDPE	17.6–35.8 ng/g	China	Shi et al. (2009)
	TBBPA-BDBPE	17.3–60.4 ng/g	China	Shi et al. (2009)
Sewage sludge	BTBPE	0.31–1.66 ng/g	China	Shi et al. (2009)
	BTBPE	Average 0.7–1.4 ng/g	Norway	Nyholm et al. (2013)
	DBDPE	32 and 52 ng/g	Sweden	Kierkegaard et al. (2004)

Table 2 (*continued*)

Sample	Compounds	Conc. range	Region	Reference
	DBDPE	7.7–31 ng/g	Australia	Ricklund et al. (2008)
	DBDPE	ND – 65 ng/g	Canada	Ricklund et al. (2008)
	DBDPE	39–140 ng/g	China	Ricklund et al. (2008)
	DBDPE	6–140 ng/g	Czech rep.	Ricklund et al. (2008)
	DBDPE	34–63 ng/g	UK	Ricklund et al. (2008)
	DBDPE	70–220 ng/g	Germany	Ricklund et al. (2008)
	DBDPE	5.1–31 ng/g	New Zealand	Ricklund et al., 2008
	DBDPE	55 ng/g	South Africa	Ricklund et al. (2008)
	DBDPE	73–160 ng/g	Switzerland	Ricklund et al. (2008)
	DBDPE	54 ng/g	Sweden	Ricklund et al., 2008
	DBDPE	1.4–160 ng/g	USA	Ricklund et al. (2008)
	DBDPE	266–1995 ng/g	China	Shi et al. (2009)
	DBDPE	Average 1.9–6.3 ng/g	Norway	Nyholm et al. (2013)
	BEH-TEBP	57–515 ng/g	USA	Covaci et al. (2011)
	TBP-AE	< 0.005–0.091 μg/g	Germany	Harju et al. (2009)
	TBP	< 0.3–0.9 ng/g	Sweden	Öberg et al. (2002)
	TBBPA-BDBPE	238–8946 ng/g	China	Shi et al. (2009)
Ice core	BTBPE	ND – 5.1 pg/cm^2/yr	Svalbard	Hermanson et al. (2010)
	DBDPE	ND – 3.6 pg/cm^2/yr	Svalbard	Hermanson et al. (2010)
	PBEB	ND – 0.23 pg/cm^2/yr	Svalbard	Hermanson et al. (2010)
Water	BTBPE	ND – 2.69 pg/L	Canada	Law et al. (2006)
	BTBPE	Avearage 0.02 ng/L	China	Wu et al. (2010)
	BTBPE	Average ND – 1 ng/L	Norway	Nyholm et al. (2013)
	DBDPE	Average ND – 5.1 ng/L	Norway	Nyholm et al. (2013)
	BEH-TEBP	ND – 1.3 pg/L	Greenland	Möller et al. (2011)
	PBEB	Average 0.06 ng/L	China	Wu et al. (2010)
	PBT	Average 0.03 ng/L	China	Wu et al. (2010)
	HBB	Average 0.52 ng/L	China	Wu et al. (2010)
	HBB	ND – 0.003 pg/L	Greenland	Möller et al. (2011)
	TBP	0.378–20.2 ng/L	Korea	Sim et al. (2009)
	TBP	ND – 6 ng/L	Germany	Reineke et al. (2006)
	TBBPA-BDBPE	Average ND – 18 ng/L	Norway	Nyholm et al. (2013)
Plankton	BTBPE	ND – 3.72 mg/g lipid	Canada	Law et al. (2006)
	DBDPE	ND – 1.51 mg/g lipid	Canada	Law et al. (2006)
Algae	TBP	4.5–68 ng/g	Australia	Whitfield et al. (1992)
Mushrooms	TBP	0.22–240 ng/g	Australia	Whitfield et al. (1992)
Prawn	BTBPE	Average 44.7 ng/g lipid	China	Wu et al. (2010)
	DBDPE	Average 84.3 ng/g lipid	China	Wu et al. (2010)
	PBEB	Average 6.35 ng/g lipid	China	Wu et al. (2010)
	PBT	Average 1.55 ng/g lipid	China	Wu et al. (2010)
	HBB	Average 197 ng/g lipid	China	Wu et al. (2010)
	TBP	Average 7.8–97 ng/g	Australia	Whitfield et al. (1992)
Bivalvia	BTBPE	ND – 303 ng/g lipid	USA	La Guardia et al. (2012)
	BEH-TEBP	ND – 1370 ng/g lipid	USA	La Guardia et al. (2012)
Fish	BTBPE	ND – 0.84 mg/g lipid	Canada	Law et al. (2006)
	BTBPE	< 0.012–0.15 ng/g lipid	China	Shi et al. (2009)
	BTBPE	1.71–518 ng/g lipid	China	Wu et al. (2010)
	DBDPE	ND – 2.71 mg/g lipid	Canada	Law et al. (2006)
	DBDPE	ND – 338 ng/g lipid	China	Wu et al. (2010)
	PBEB	3.98–25.6 ng/g lipid	China	Wu et al. (2010)
	PBT	1.2–3.24 ng/g lipid	China	Wu et al. (2010)
	HBB	680–2451 ng/g lipid	China	Wu et al. (2010)
	TBP	6–171 ng/g	Brazil	Oliveira et al. (2009)
	TBP	ND – 3.4 ng/g	Australia	Whitfield et al. (1995)
Shark	BTBPE	ND – 8.1 ng/g lipid	Iceland	Strid et al. (2013)
	PBEB	ND - 13 ng/g lipid	Iceland	Strid et al. (2013)
Whales	PBEB	< 0.11–6.68 ng/g lipid	USA	Montie et al. (2010)
	PBT	< 0.11–0.68 ng/g lipid	USA	Montie et al. (2010)
	HBB	< 0.11–3.12 ng/g lipid	USA	Montie et al. (2010)
Seal	PBEB	< 0.02–3.73 ng/g lipid	USA	Montie et al. (2010)
	TBP-AE	3.1–9.1 ng/g	Barents sea	von der Recke and Vetter (2007)
Panda	DBDPE	ND – 863 ng/g lipid	China	Hu et al. (2008)
Bird eggs	BTBPE	< 0.02–0.17 ng/g	Faroe Islands	Karlsson et al. (2006)
	BTBPE	ND – 0.96 ng/g	Norwegian arctic	Verreault et al. (2007)
	BTBPE	0.04–0.7 ng/g	USA	Gauthier et al. (2007)
	DBDPE	ND – 1.7 ng/g	China	Gao et al. (2009)
	PBEB	0.03–0.23 ng/g	Norwegian arctic	Verreault et al. (2007)
	PBEB	0.03–1.4 ng/g	USA	Gauthier et al. (2007)
	PBT	ND – 0.12 ng/g	Norwegian arctic	Verreault et al. (2007)
	PBT	0.004–0.02 ng/g	USA	Gauthier et al. (2007)

Table 2 (*continued*)

Sample	Compounds	Conc. range	Region	Reference
	HBB	0.42–2.64 ng/g	Norwegian arctic	Verreault et al. (2007)
	HBB	0.24–0.53 ng/g	USA	Gauthier et al. (2007)
	TBBPA-BDBPE	ND – 0.36 ng/g	Canada	Letcher and Chu (2010)
Bird	BTBPE	0.07–2.41 ng/g lipid	China	Shi et al. (2009)
	BTBPE	ND - 0.26	Norwegian arctic	Verreault et al. (2007)
	DBDPE	9.6–124 ng/g lipid	China	Shi et al. (2009)
	DBDPE	ND – 800 ng/g	China	Luo et al. (2009)
	PBEB	ND – 0.12 ng/g	USA	Venier et al. (2010)
	PBT	ND – 0.15 ng/g	Norwegian arctic	Verreault et al. (2007)
	HBB	ND – 0.15 ng/g	Norwegian arctic	Verreault et al. (2007)
Human	HBB	2.1–4.1 ng/g	Japan	Yamaguchi et al. (1988)
	TBP	0.17–81 ng/g lipid	Norway	Thomsen et al. (2001b)
	TBP	ND – 130 pg/g	Japan	Kawashiro et al. (2008)
Wine	TBP	3.6–392.6 ng/L	Several winery	Chatonnet et al. (2004)

BTBPE was also found in the eggs and plasma of the northern nulmar (*Fulmarus glacialis*) in Faroe Islands (Karlsson et al., 2006) and in the egg yolks and plasma of male and female glaucous gulls (*Larus hyperboreus*) in the Norwegian Arctic (Verreault et al., 2007). This documents that the compound undergoes long-range atmospheric transport, bioaccumulates in the birds and is maternally transferred (to eggs). The results reported above reflect the extensive use of BTBPE, as well as its long-term persistency in the environment but, until now, this BFR has so far not attracted sufficient attention from the scientific community.

In a recent study (Wu et al., 2011), the BTBPE concentrations in seawater and in marine organisms were used to calculate the bioaccumulation factor (log BAF): the values ranged from 3.32 to 6.08, depending on the individual species. Tomy et al. (2007) exposed juvenile rainbow trout (*Oncorhynchus mykiss*) to environmentally relevant doses of BTBPE for 49 days and reported a linear rate of absorption of 6.9 pmol/day. The biomagnification factor was set at 2.3, thus indicating a high potential for this compound to magnify in aquatic food webs. The same authors also attempted to identify BTBPE metabolites in trout liver tissues, but were not successful. Another study of the metabolism of ^{14}C BTBPE in rats showed that 99% of the total sum of the ^{14}C was excreted in the feces and 1% was excreted in the urine (Hakk et al., 2004). Radioactivity was also observed following administration in all the tissues except the brain. The authors pointed out that BTBPE is poorly absorbed in the gut following oral administration and more than 94% of the BTBPE was excreted by the tissues with minimum delays (Hakk et al., 2004). La Guardia et al. (2012) detected BTBPE in filter-feeding bivalve (*Corbicula fluminea*) and grazing gastropods (*Elimia proxima*) in downstream of a North Carolina WWTP outfall. The authors recorded lower potential for accumulation than for PBDEs, where BAF and the biota-soil accumulation factor (BASF), were generally an order of magnitude lower than those of detected for PentaBDEs. Ma et al. (2013) monitored BFRs in the atmosphere of the Great Lakes and the concentrations of BTBPE were unchanged or decreased with half times of ~4 years in all three phases (vapor, particles, precipitation) at all the monitored sites. Recently, BTBPE was detected in Greenland shark liver from the Iceland area at a mean concentration 0.61 ng/g fat (Strid et al., 2013). The authors tried to compare the detected concentrations with those of PBDEs, PCBs and several other halogenated pollutants; however, BTBPE did not correlate with any other compounds.

BTBPE has low acute toxicity in model organisms. Harju et al. (2009) reported lethal concentrations greater than 36.7 g/m^3 in rats after BTBPE inhalation for period of 4 hours; the only relevant effects observed were behavioral modification, gastrointestinal lesions and dermatitis. Dermal application to rabbits established a lethal dose greater than 10 g/kg with nutritional and metabolic changes (Harju et al., 2009). BTBPE mutagenicity was assessed using *Salmonella typhimurium* strains TA98, TA100, TA 1535, TA1537 and TA1538 and *Saccharomyces cerevisiae* strain D4, both in the presence and in the absence of metabolic activation. BTBPE did not cause any positive reaction in any test (Harju et al., 2009). The hormonal activities of this compound have been investigated with the result that BTBPE may cause antiestrogenic effects (Ezechiáš et al., 2012).

Very little information is available on BTBPE biodegradation in the environment. The compounds belong among less brominated BFRs in terms of possible anaerobic microbial dehalogenation, which is usually more pronounced for higher degree of halogenation (Vrkoslavová et al., 2011). An aerobic attack at the etheric bond, which could lead to the formation of less recalcitrant TBP, as is known for PBDEs (Kim et al., 2007), is more probable. Due to the presence of bromine in the *para* positions, another possible transformation route could be hydroxylation with monooxygenase enzymes, as was documented by Hakk et al. (2004) in rats. This mechanism is probable even in other compartments of the environment (e.g. soil) due to the presence of the cytochrome P450 enzymes in fungi, which are able to transform halogenated pollutants (Cajthaml, 2014)

BTBPE is an emerging pollutant that is often found in the various environmental compartments, but further scientific research is required to identify potential environmental risks. According to the available information, there is no legislative restriction on the use of this substance in any country.

3.2. Decabromodiphenylethane (DBDPE)

DBDPE began to emerge on the market in the 1990s as an alternative to DecaBDE (BDE209) and is sold under various brand names, such as SAYTEX 8010 or Milebrome 8010. DBDPE is used in the same products as previously contained DecaBDE, namely ABS, polycarbonate/ABS, HIPS/polyphenylene oxide and textiles (Harju et al., 2009).

DBDPE has been determined in products such as insulating tubes for water pipes at an approximate concentration of 4.8 mg/g. This value is substantially lower than the expected value for HIPS material (12% wt:wt) and lower than other BFRs (*e.g.* BDE209) probably due to less than optimum extraction (Kierkegaard et al., 2004). DBDPE has also been detected in household dust from different environments, with average concentrations of 270 ng/g for homes, 170 ng/g for offices and 400 ng/g for cars in the United Kingdom (Harrad et al., 2008). Other studies also confirmed the presence of this substance in household dust in a wide range of concentrations, from less than 10 to 11070 ng/g in the USA (Stapleton et al., 2008). Karlsson et al. (2007) and Ali et al. (2011) again documented a concentration in household

dust on an average of 47 ng/g in Sweden and 293 ng/g and 789 ng/g in the UK and Belgium, respectively. The high levels of this substance detected in all of the works reported above reflect its exstensive use in various products. DBDPE has also been determined in indoor air (in Sweden) at a concentration of 0.7 ng/m^3 (Kierkegaard et al., 2004) and a concentration of 0.0229 ng/m^3 (Karlsson et al., 2007). Gorga et al. (2013) performed a study in which they analyzed several new emerging BFR including DBDPE in sewage sludge from a number of Catalonian treatment plants. Significant concentrations of DBDPE were detected upto 257 ng/g dw, probably due to its increasing production as a Deca-BDE substitute. The ratios between BDE-209 and DBDPE with a mean value of 5.1, indicates that the presence of DBDPE in sewage sludge should not be overlooked.

DBDPE concentratios in a sediment sample from the Netherlands was determined at a concentration of 24 mg/kg (Kierkegaard et al., 2004). DBDPE was also detected in the sludges of Swedish wastewater treatment plants, at concentrations of 52 ng/g and 32 ng/g (Kierkegaard et al., 2004). Ricklund et al. (2008) monitored the presence of this flame retardant in sludges from various countries and concluded that DBDPE contamination is rather widespread in Europe. The highest concentration was observed in Germany (216 mg/kg dry weight) (Ricklund et al., 2008; Covaci et al., 2011). In spite of these observations, DBDPE is not detected as often in biological matrices. In a study of the food chain in Lake Winnipeg, no detectable DBDPE values were found in zooplankton and mussels, while 1 mg/kg fat was detected in walleye (*Sander vitreus*) (Law et al., 2006). Another study concerned with various species of aquatic birds living near a recycling facility for electronic waste in China. DBDPE was detected in all the samples of bird tissues except one, with a maximum concentration of 124 ng/g (Shi et al., 2009) and 800 ng/kg of lipid weight (Luo et al., 2009). DBDPE was also measured in various mammal tissues. Various body organs of captive giant and red pandas were analyzed for DBDPE and the substance was found in most samples. The maximum concentration detected was detected in the gonad tissues (863 ng/g fat) (Covaci et al., 2011). All the results obtained so far suggest DBDPE biomagnification in the food chain. Biomagnification factors (BMF) for this BFR in the aquatic food chain of Lake Winnipeg were in the range 0.2–9.2 (Law et al., 2006). For DBDPE, also a high BAF value with log BAF values between 6.1 and 7.1 has also been reported (He et al., 2012). Zhang et al. (2013) determined BASF of DBDPE using oligochaete *Lumbriculus variegates*. The authors determined BASF using a 49-day exposure experiment and found out a good agreement of this value with calculated BASF by dividing uptake and elimination rate constants. The authors found an inverse correlation between the uptake rate constant and log K_{ow}.

It was found that this flame retardant is not acutely toxic (Harju et al., 2009). Single-dose and long-term (90 days) experiments in rats yielded a lethal dose (LD_{50}) of greater than 5000 mg/kg body weight. At such high doses, changes in the liver weight and histomorphological effects were observed. The acute dermal toxicity was measured in rabbits, yielding LD_{50} greater than 2000 mg/kg body weight (Harju et al., 2009). On the other hand, Johnson et al. (2013) found a positive correlation between the DBDPE concentrations in dust and triiodothyronine levels in the exposed men, which can indicate a disruption of thyroid hormonal signals.

Unfortunately, not many reports have been published about the biodegradability of this flame retardant, but a report from UK EPA suggested that DBDPE degradation in the air *via* OH radicals, in aquatic environments *via* hydrolysis and in wastewater treatment plants (WWTPs) is not likely to occur rapidly (Harju et al., 2009). A key step in the DBDPE biotransformation is probably bacterial dehalogenation under anaerobic conditions (Janssen et al., 2001). During this step, the halogen substituent, which is usually responsible for the recalcitrant character of the compound, is replaced – often by hydrogen or a hydroxyl group. However, scientific evidences for DBDPE debromination is still lacking.

3.3. Bis(2-ethylhexyl) tetrabromophtalate (BEH-TEBP)

BEH-TEBP is another member of the NBFRs family and is used mainly as an additive flame retardant /plasticizer in the production of polyvinyl chloride (PVC) and neoprene. Other relevant applications include cables and wire insulation, foil pads under carpets, fabrics, wall coatings and adhesives (Covaci et al., 2011). BEH-TEBP together with 2-ethylhexyl 2,3,4,5-tetrabromobenzoate (EH-TBB), forms part of a commercial mixture (FIREMASTER 550) which is used to replace PentaBDE (Stapleton et al., 2008). It is stated that EH-TBB and BEH-TEBP are present in this mixture in a ratio of 4:1. However, BEH-TEBP is also included in other commercially available mixtures.

BEH-TEBP was detected in sludge from WWTPs in San Francisco in the range 57–515 ng/g (Covaci et al., 2011). It was also determined in indoor dust in British and Belgian cities: the average concentrations were 381 ng/g for classrooms, 212 ng/g for households and 95 ng/g for offices (Ali et al., 2011). Stapleton et al. (2008) also reported the presence of BEH-TEBP in all the analyzed US indoor dust samples, with reported geometric means of 234 ng/g for living rooms and 105 ng/g for bedrooms. BEH-TEBP concentrations were found to be on the increase in three of the five Great Lakes atmosphere samples with a doubling time of ~2 years (Ma et al., 2013). These results correspond to the current exstensive use of this compound in the commercial flame retardant market.

In the above mentioned work La Guardia et al. (2012) also detected BEH-TEBP in filter-feeding bivalve and grazing gastropods in the USA. The values of BAF and BASF were similar to those of BTBPE and generally an order of magnitude lower than those of the detected PentaBDEs.

Bearr et al. (2010) tested the mixtures Firemaster® 550 and Firemaster® BZ-54 (1 mg/fish/day) mixtures, which exhibited acute genotoxicity with DNA damage observed in liver cells. Johnson et al. (2013) recorded a positive correlation between BEH-TEBP concentrations in dust and triiodothyronine levels in the exposed men suggesting possible endocrine disrupting activity. However, the toxic effects of BEH-TEBP on humans and other model organisms have been ignored, as have its (bio)degradation and fate in the environment. BEH-TEBP did not exibit any activation of the aryl hydrocarbon receptor in the H4IIE assay or agonistic effects on the estrogenic and androgenic receptors using recombinant yeast (Saunders et al., 2013). BEH-TEBP produced maximum anti-androgenic effects of 74% at 300 mg/L assayed with recombinant yeast. Significant effects were also observed in the H295R assay where at 15 mg/L, BEH-TEBP exposure resulted in a 5.4-fold increase in the concentrations of E2. These results clearly document the endocrine disrupting properties of BEH-TEBP.

To date, little is known about the possible degradation of this substance in the environment. However, Davis and Stapleton (2009) studied the photodegradation of several BFRs and concluded that BEH-TEBP undergoes photodissociation to form di- and tribrominated analogs (half-lives in the range 168.4–220.2 min). The same authors also reported that 2-ethylhexyltetrabromobenzoate is subject to photodegradation faster than BEH-TEBP (half-lives of 85. and 162.3 min, respectively), a fact which could explain the different ratios of these substances in commercially available mixtures and therefore in household dust. Typically, phthalic esters are transformed *via* hydrolysis of the esteric bond (Liang et al., 2008). However, the biodegradability difference for phthalates is probably due to the steric effect of phthalates ester side chains, which hinders the hydrolytic enzymes in binding to the phthalates and thereby inhibits their hydrolysis. Despite the fact the problem could be even more pronounced for BEH-TEBP due to the high substitution with

bromine atomes, de Jourdan et al. (2013) detected hydrolysis products originating from either from biotic or abiotic processes.

3.4. Tetrabromophthalic anhydride (TEBP-Anh)

TEBP-Anh is an alternative additive and is used as a reactive flame retardant in unsaturated polyesters, polyurethane foams, paper, textile, wool and epoxides (Tice and Masten, 1999). It is sold under various brand names such as Bromphthal, FIREMASTER PHT 4 or Saytex RB 49.

TEBP-Anh was found in soil mainly in its hydrolyzed form i.e. tetrabromophthalic acid (Tice and Masten, 1999). Scant volatilization from the soil was observed, whilst the majority of the compound tended to absorb into the soil particles. Interestingly, it was found to have a slight positive effect on soil bacteria density upon spiking with TEBP-Anh (10 μg/g f.c.). Soil fungi were not significantly affected by this substance (Tice and Masten, 1999). Under environmental conditions and after exposure to UV radiation, TEBP-Anh is rapidly hydrolyzed (half-life of less than 5 min) to form a dicarboxylic acid (Tice and Masten, 1999). The concentration of this compound was below the detection limit in various sediment samples (Covaci et al., 2011).

Oral administration was tested in rats to determine the toxicological features of TEBP-Anh (Tice and Masten, 1999). It was found that TEBP-Anh is rapidly hydrolyzed to the corresponding acid derivative in rats. TEBP-Anh was partly absorbed in the gastrointestinal tract and then rapidly eliminated in the urine (almost 20% of the absorbed dose within 24 h) mostly as an acid derivative. The unabsorbed portion was excreted *via* the feces (almost 75% within 48 h). In addition, the pharmacokinetics of TEBP-Anh were observed to be the same in both sexes. After 48 h, the total residues in all the tissues were $<0.2\%$ of the administered dose; the rate elimination constant ranged from 0.211 to 0.100 and the retention half-life was <7 h (Tice and Masten, 1999). In view of these results, it is not anticipated that TEBP-Anh would accumulate in the human body (Tice and Masten, 1999).

It was found that TEBP-Anh has very low acute oral toxicity, with LD_{50} higher than 10,000 mg/kg in rats. In addition, no teratogenic effects were observed (Tice and Masten, 1999). Mutagenicity tests were performed on in *Salmonella typhimurium* strains TA98, TA100, TA 1535, TA1537, TA1538, and as well as in *Saccharomyces cerevisiae* strain D4. TEBP-Anh – at doses ranging from 0.1 to 10.000 μg/plate – did not exhibit any mutagenic activity either in the presence or in the absence of metabolic activation (Tice and Masten, 1999). On the other hand, Brown et al. (2004) demonstrated the ability of the commercial mixture FIREMASTER PHT4 (where TEBP-Anh is the main component) to activate the aryl hydrocarbon receptor (AhR) under *in vitro* conditions.

Due to its rapid hydrolyzation (Tice and Masten, 1999), little is known about the presence of this novel flame retardant and especially about its possible transformation products in various environmental compartments. Bacterial dioxygenases are very substrate-specific and therefore it is unlikely that TEBP-Anh can be transformed by related enzymes e.g. *ortho*-cleavage dioxygenases after respective decarboxylation. Moreover it is noteworthy that TEBP-Anh belongs among organopollutants present at very low concentrations and therefore enhanced evolution of specific degradative genes is also not probable (Furukawa and Fujihara, 2008). Further research is therefore needed to address these issues.

3.5. 2,3,4,5,6-pentabromoethylbenzene (PBEB)

PBEB is used in textiles, adhesives, polyurethane foam, polyester resins and paints and as an additive for unsaturated polyesters (WHO, 1997). It was produced in the USA mainly in the 1970s and 1980s under the trade name FR-105. Current production figures are not publicly available; however, it is not produced in any country signatory to the OSPAR convention (Covaci et al., 2011; de Wit et al., 2010; Hoh et al., 2005).

PBEB was detected in the air, bound to aerosol particles and in the gas phase at concentrations of 29 and 520 pg/m^3, respectively (Hoh et al., 2005). A recent study of the atmospheric deposition of BFRs in polar regions revealed that a slight increase in the PBEB concentration has occurred since the early 1970s; however PBEB was still detected at lower levels than other known BFRs (*i.e.* DecaBDE and HBCD) (Hermanson et al., 2010).

Wu et al. (2011) compared the concentration of PBEB in seawater and in various marine organisms and calculated the corresponding log BAF, which ranged from 2.72 to 4.09, depending on the individual species. This flame retardant was also determined in the tissues of birds from the Canadian Great Lakes region (Venier et al., 2010; Gauthier et al., 2007). In particular, PBEB was detected in two plasma samples from bald eagles (*Haliaeetus leucocephalus*) with a mean concentration of 0.11 ng/g. The same compound was found in herring gull (*Larus argentatus*) eggs in concentrations ranging from 0.03 to 1.4 ng/g. PBEB was also detected in the fat tissues of harp seals (*Pagophilus groenlandicus*), hooded seal (*Cystophora cristata*) and whales (*Eubalaena glacialis*) in the range 0.02–6.68 ng/g fat (Montie et al., 2010). Moreover, PBEB was detected in 13 of 19 samples of river sediments collected near Barcelona (Spain) at concentrations ranging from 3.1 to 10 ng/g dry weight (Guerra et al., 2010). This substance has not yet been detected in human blood plasma. An in-depth analysis of the BFR content in plasma samples from various groups of Chinese people ($n=128$) detected several brominated compounds, but not PBEB (Zhu et al., 2009). On the other hand PBEB was detected in the livers (3.0 ng/g fat) of Greenland shark (Strid et al., 2013).

PBEB has been tested for acute toxicity in rabbits and LD_{50} values greater than 8 g/kg were reported (Harju et al., 2009). No mutagenic activity was observed for this compound according to the *S. typhimurium* assays (Harju et al., 2009). No other adverse effects, such as endocrine-disrupting or metabolic activity of this substance, have been reported.

This flame retardant is considered to be an alternative to PBDEs and, despite to its frequent detection in environmental samples, it is still not clear whether its presence in environmental matrices is due to increased use in recent years or to persistence of the substance in the environment. To the best of our knowledge, there is no available literature describing the biotransformation process of PBEB and PBT, that is discussed in the following section. Both of the compounds are heavily substituted with bromines and therefore anaerobic dehalogenation is the first necessary step as well as in the case of hexachlorobenzene (Field and Sierra-Alvarez, 2008). However, reductive dehalogenation where halogens acted as terminal acceptors of electrons has not been documented. Further research is necessary, specifically in relation to toxicity for humans and the fate of this substance in the environment.

3.6. 2,3,4,5,6-pentabromotoluene (PBT)

PBT is another compound of the group of alternative BFRs. PBT is used as an additive in unsaturated polyesters, mainly for electrical equipment, and in polyethylene, polypropylene, polystyrene, textile, and ABS (Covaci et al., 2011). Production amounts are estimated to be between 1000 and 5000 t per year and it is sold under brand names such as Flammex or FR-105 (Covaci et al., 2011).

It has been reported that this flame retardant has the potential for long-range transport in the atmosphere, like PBDE (de Wit et al., 2006). This compound was detected in the air over the Greenland Sea in concentrations ranging from 0.001 to 0.02 pg/m^3

in the gaseous phase and up to 0.001 pg/m^3 bound to suspended particles. Moreover, PBT was detected in all the air samples from the East Greenland Sea (Möller et al., 2011). Other investigators also detected PBT in one air sample from Egbert (ON, Canada) at a concentration of 0.02 pg/m^3, while the values in the other samples were below the detection limit (0.01 pg/m^3) (Gouteux et al., 2008). The same compound was also identified in sediments of the river Elbe River, where concentrations ranged from less than 1 to 25 ng/g dry weight (Schwarzbauer et al., 2001).

PBT log BAF in several water organisms are in the range 2.04–4.77, depending on the species (Wu et al., 2011). Relative concentrations of this BFR in various organisms of the marine food chain reached values of up to 1.2–3.6 ng/g fat (Wu et al., 2010). The same authors also observed a reduction in the PBT concentration with every gradual increase in the trophic level of the alimentary chain. PBT was detected in the plasma and eggs of the glaucous gull (*L. hyperboreus*) and the highest concentrations reported were 0.15 ng/g for males, 0.06 ng/g for females and 0.12 ng/g in egg yolks (Verreault et al., 2007). Gauthier et al. (2007) also detected PBT in herring gull eggs (*L. argentatus*) at concentrations ranging from 0.004 to 0.02 ng/g wet weight; however, it should be noted that, in this study, the PBT concentrations were the lowest among all the BFRs investigated. The flame retardant was also detected in the fatty tissues of harp seals (*P. groenlandicus*), hooded seals (*C. cristata*) and whales (*E. glacialis*) at concentrations of less than 0.02–0.68 ng/g fat (Montie et al., 2010).

Toxicology studies were conducted on rats, where PBT was administered orally at doses ranging from 0.05 to 500 mg/kg for 91 days. There were no evident signs of PBT toxicity, except some histological changes in the thyroid gland, liver and kidneys. The no observed adverse effect level (NOAEL) was set at 0.35 mg/kg body weight per day (Harju et al., 2009). The lethal concentration for fish was found to be greater than 5 mg/L (Harju et al., 2009). There was no observed mutagenic activity as assessed by *S. typhimurium* assays, either in the presence or in the absence of metabolic activation (Harju et al., 2009). As already reported for other NBFRs (*e.g.* PBEB), PBT is able to bind and activate the Ah receptor (Brown et al., 2004). Moreover, binding of the substrate to the receptor stimulated AhR-dependent gene expression at high levels. However, it should be noted that the PBT-AhR affinity is significantly lower than that reported for other BFRs, namely TEBP-ANH and DecaBDE (Brown et al., 2004).

Overall, limited information is available about the fate of this new flame retardant in the environment and about its possible negative impact on human health; further research work is needed to fill these knowledge gaps.

3.7. Hexabromobenzene (HBB)

HBB is another member of the NBFR family and is used as an additive flame retardant in paper, wool, textiles, electronics and plastics (Covaci et al., 2011). HBB is produced mainly in Eastern Asia, but there is no record about its production in European countries (Covaci et al., 2011). The Chinese corporation Shou Guang Longfa Chemical Co. Ltd. produces 600 t p.a. of HBB and in Japan, HBB is marketed by the Japanese Nippoh Chemicals Corp. under the trade name FR-B (Covaci et al., 2011).

Hexabromobenzene is frequently determined in samples from the polar regions, providing evidence for its long-range transportation in the atmosphere. Möller et al. (2011) detected HBB in all the studied atmospheric samples, both in the gas phase and bound to aerosol particles. Its measured concentrations ranged between 0.04 and 0.66 pg/m^3 for the gaseous phase and from 0.001 to 0.005 pg/m^3 bound to particles. Significantly lower HBB levels (0.02–0.09 pg/m^3) were detected in air samples by Gouteux et al. (2008). In this study, it was also demonstrated that heating of commercially available mixtures promoted HBB volatilization at a rate of 100 ng/g/h. Nevertheless, the highest concentrations of HBB were found in the atmosphere in the People's Republic of China, where its concentration reached up to 6.5 pg/m^3 (Qiu et al., 2010). The same authors also reported an average amount of 7.7 pg/L HBB in the water of Tahiu Lake.

It is important to note that, once HBB enters the environment, the compound has the potential to accumulate in organisms. Belfroid et al. (1995) measured the biomagnification factor (BMF), expressed as the HBB concentration in earthworm/HBB concentration in food, for the earthworm *Eisenia andrei*: regardless of the amount of HBB in the diet, BMF values were invariably lower than 0.17 suggesting either low or no biomagnification. However, Wu et al. (2011) measured BAF using aquatic organisms and, 5 out of 6 cases, the log BAF value was above 3.7, documenting high bioaccumulative properties. The same compound was detected in the plasma of wild glaucous gulls (*L. hyperboreus*) at a maximum concentration of 0.15 ng/g (wet weight). The authors noted that the highest concentrations were detected in samples from male birds (Verreault et al., 2007). In glaucous gull (*L. hyperboreus*) eggs, HBB was detected in all the samples in concentrations ranging from 0.42 to 2.64 ng/g. Another study also found that this retardant is abundant in eggs. Gauthier et al. (2007) detected concentrations ranging from 0.24 to 0.53 ng/g in the yolks of herring gull (*L. argentatus*) eggs. Hexabromobenzene was also detected in human tissues at concentrations ranging from 2.1 to 4.1 ng/g wet weight. Other less brominated benzene derivatives were also identified as putative metabolic products of HBB (Harju et al., 2009).

The lowest toxic dose established for orally administered HBB in rats was 150 mg/kg. Dietary absorption of higher doses for 12 weeks gave rise to effects on the liver, enzyme inhibition and induction of, or changes in, blood or tissue levels (Harju et al., 2009). There were no histopathological changes when HBB was administered to mice at 20–90% of the lethal dose, but it reduced the liver glutathione levels and increased the gamma-glutamyltransferase activity in serum and the concentration of malondialdehyde in the liver (Szymanska, 1998). The lowest toxic dose determined for oral exposure in birds was 1.5 g/kg/15 days for quail and 52.5 g/kg/12 weeks for chickens. The effects were similar to those observed for rats (Harju et al., 2009). No teratogenic effects were demonstrated for rats upon administration of 200 mg/kg HBB body weight on the 5th and 15th day of gestation (Harju et al., 2009). HBB induced an increase in the porphyrin level in rats and therefore this substance is reported as a porphyrinogen (Szymanska and Piotrowski, 2000). A wide range of concentrations (10–10,000 μg/plate) were tested for mutagenicity using *Salmonella typhimurium* strains TA98, TA100, TA 1535, TA1537, with or without metabolic activation. All the tests yielded negative results (Harju et al., 2009). HBB was also demonstrated to have AhR-binding and -activation features (Brown et al., 2004). The results show that high concentrations of HBB activate AhR-dependent gene expression at similar levels to those for DecaBDE. Nevertheless, Larsson et al. (2006) demonstrated that HBB is not an agonist or antagonist of the androgen receptor in human liver cells.

Very little is known about possible transformation processes. Nyholm et al. (2010) studied the transformation of HBB in soil under aerobic and anaeriobic conditions. HBB degraded rapidly (with half-life of less than 40 days) in aerobic soil and, surprisingly, much more slowly under anoxic conditions, with a half-life of more than 100 days. The degradation results under aerobic conditions were even better when the authors amended the soil with 0.5% activated sludge. However, the authors did not perform any further analysis of the degrading microbial community.

3.8. 2-Allyloxy-1,3,5-tribromobenzene (TBP-AE)

This flame retardant is also known in the literature as allyl 2,4,6-tribromophenyl ether (Harju et al., 2009). TBP-AE is sold

under the brand names PHE-65, milebrome TBPAE and others. TBP-AE is used either as a reactive BFR, when it is incorporated at the stage of polymerization, or just as additive BFR, mainly in expanded polystyrene (EPS) and polystyrene foam (Covaci et al., 2011). TBP-AE was produced in Germany from the 1980s and is still produced in the USA (227 t/year in 2006) (Covaci et al., 2011).

According to the methodology developed by Brown and Wania (2008), TBP-AE has dissociation properties which suggest it could potentially become an Arctic pollutant. In addition, the compound possesses similar properties to those of other known Arctic contaminants. It is worth noting that degradation of TBP-AE during WWTP processes is probably limited (Harju et al., 2009). This brominated compound was detected in 15 out of 18 samples of sludges collected from municipal sewage treatment plants in Germany. The highest concentration detected was 0.091 mg/kg dry weight (Harju et al., 2009).

TBP-AE was found in the fat and brain tissues of harp seals (*P. groenlandicus*) and hooded seals (*C. cristata*) from the Barents Sea in concentrations ranging from 5.4 to 9.1 ng/g for the latter and from 3.1 to 10 ng/g for the former (von der Recke and Vetter, 2007). Experiments were performed under anaerobic conditions using super-reduced corrinoids that transformed 2,3-dibromopropyl-2,4,6-tribromophenyl ether (DPTE) to TBP-AE and 2-bromoallyl-2,4,6-tribromophenyl ether (BATE). Therefore, the simultaneous occurrence of TBP-AE and BATE in fat and brain tissue of mammals is assumed to be caused by metabolic transformation of DPTE (von der Recke and Vetter, 2007).

The presence of an etheric bond and the number of substituents are similar to BTBPE. Therefore, also here, possible degradation routes are: aerobic attack at the etheric bond (Kim et al., 2007), hydroxylation with monooxygenase enzymes (Hakk et al., 2004) and, in this case, also possible reaction of the alkenyl double bond. However this novel BFR has not been sufficiently studied to date from the point of view of transformations in the environment. In addition, the adverse effects of TBP-AE on humans and wildlife need to be addressed and its fate in the various environmental compartments still remains unknown.

3.9. 2,4,6-Tribromophenol (TBP)

This substance has already been well known for years and has been the subject of a number of scientific communications. TBP is marketed under various trade names, such as PH-73FF, FR-613 and others. TBP is currently used as (i) an antiseptic and germicidal agent in pharmaceutical preparations, (ii) a flame retardant in thermoplastic polyesters, epoxy resins, polystyrene and ABS, (iii) a fungicide in wood treatment processes and (iv) a monomer for the synthesis of other substances, such as BTBPE (Covaci et al., 2011; Harju et al., 2009; Mardones et al., 2008). Nevertheless, TBP is also produced in small amounts in natural environments by marine algae (Flodin and Whitfield, 1999). Production of TBP in the USA amounted to 23,000 t in 2006. In 2001, 2500 t were produced in Japan and 9500 t worldwide (Contreras et al., 2009).

It is worth noting that TBP is one of the most volatile flame retardants and can thus be expected to be present in indoor air. Thomsen et al. (2001a) confirmed this hypothesis by analyzing air samples in their laboratory while working with BFRs; TBP indeed ranked among the most abundant brominated compounds. TBP was also determined in dust at concentrations from 16 to 130 ng/g in households and from 27 to 620 ng/g in offices in Japan (Takigami et al., 2009). This flame retardant is also frequently reported in water. When 22 samples of sludges from Swedish wastewater treatment plants were analyzed, the highest detected concentration of TBP was 0.9 ng/g wet weight (Öberg et al., 2002). In a recent study carried out on samples collected along the Korean coast, TBP concentrations were found in the range 0.378–20.2 ng/L in seawater and 0.56–12.3 ng/g dry weight in sediments (Sim et al., 2009). Similarly, Reineke et al. (2006) screened water and sediments from the North and Baltic Seas for their contents of brominated aromatics, where TBP was detected only in water samples (maximum concentration 6 ng/L).

In a study of wildlife organisms exposed to BFRs in acquatic environments, it was found that prawns caught near Australian coasts contained TBP in average concentrations of 7.8, 8.5, 41 and 97 ng/g in different bays (Whitfield et al., 1992). Oliveira et al. (2009) studied the occurrence of bromophenol in two types of fish tissues, gut and muscles, from two members of the Lutjanidae family. The authors found that the TBP content in stomach tissues was higher than in muscles, with concentrations ranging from 15 to171 ng/g for *Lutjanus synagris* and from 6 to 119 ng/g for *Ocyurus chrysurus*. The highest TBP concentration reported among ten species of fish caught off the Australian coast was 3.4 ng/g (Whitfield et al., 1995). Interestingly, slightly higher TBP concentrations were found in carnivorous fishes, suggesting TBP biomagnification through the food chain (see below). The same compound was also detected in brown algae (14–38 ng/g wet weight), red algae (4.5–68 ng/g), bryozoa (24–27 ng/g), polyps (29 ng/g) and mushrooms (0.22–240 ng/g) collected in Australia (Whitfield et al., 1992). Surprisingly, TBP was also detected in red wine in concentrations ranging from 3.6 to 392.6 ng/L (Chatonnet et al., 2004). All these studies point to the widespread use of this flame retardant and its presence in the environment. In addition, TBP was reported to be one of 120 chemicals with chemical properties similar to known Arctic contaminants (Brown and Wania, 2008).

The adverse effects of TBP on various model organisms have been studied in detail (Harju et al., 2009). Acute oral toxicity tests on rats enabled the calculation of LD_{50} for this compound, equaling 1995 mg/kg body weight for males and 1819 mg/kg body weight for females. Higher LD_{50} values (5012 mg/kg) for both males and females were reported in another study (Harju et al., 2009). The toxic effects observed included decreased motor activity, nasal discharge, lacrimation, tremors, prostration, clonic convulsions and death. The inhalation toxicity LC_{50} was found to be more than 1630 mg/m for 4 h exposure. A study of inhalation of dust particles containing TBP yielded LC_{50} greater than 1.63 mg/L for 4 hours exposure. Another study found LC_{50} greater than 200 mg/L/h (Harju et al., 2009). Dermal administration of TBP in rabbits resulted in LD_{50} greater than 2000 mg/kg (Harju et al., 2009). In an experiment to study developmental neurotoxicity and immunotoxicity, pregnant rat females were exposed to TBP (inhaled) at concentrations from 0.03 to 1 mg/m. The results suggested that, during this exposure regime, TBP could be a developmental neurotoxicant, embryotoxicant and fetotoxicant but not immunotoxicant (Harju et al., 2009). In a study of the acute toxicity of several BFRs in *Nitocra spinipes*, Breitholtz et al. (2008) identified TBP as one of the less toxic flame retardants, with an LC_{50} of 920 mg/L.

TBP was tested for mutagenic activity using *Salmonella typhimurium* (strains TA98, TA100, TA 1535, TA1537 and TA1538) and *Saccharomyces cerevisiae* (strain D4), either in the presence or in the absence of metabolic activation. The results were negative in all cases (Harju et al., 2009). It was also suggested that TBP disrupts hormonal regulation. Olsen et al. (2002) found weak binding of this substance to the estrogen receptor (ER) by the exchange assay using 17β-estradiol. In this test, only 43% exchange was achieved at the highest tested concentrations. Another study observed an antagonistic effect on ER with $IC_{50}=8.3$ μM and a small inhibition of estradiol sulfation (Hamers et al., 2006). The same study also described the distinctive ability of TBP to replace thyroxine (T4) on the transport protein transthyretin (TTR). The ability of TBP to bind to the thyroid receptor was 10 times greater than T4 alone (Hamers et al., 2006). Moreover, Ezechiáš et al. (2012) demonstrated an antagonistic effect of TBP on androgen

and estrogen receptors. The nominal IC_{50} value was determined as 3.9 μM for androgen and 9.2 and 14.1 μM for two different estrogen assays. TBP was also studied in terms of influence on the enzyme aromatase (CYP19) with adrenal cortical cell line H295R (Canton et al., 2005). The results showed a concentration dependence of aromatase activity in the presence of TBP in the concentration range from 0.5 to 7.5 μM and thus confirmed the enzyme is influenced by this compound.

Thomsen et al. (2001b) analyzed human plasma from various Norwegian workers. Overall, the group working in an electronics dismantling facility was impacted the most by total BFRs, where TBP was the most abundant brominated compound, with concentrations in the range from 0.17 to 81 ng/g fat (Thomsen et al., 2001b). TBP was also detected in the umbilical cords of Japanese mothers at an average concentration of 33 pg/g fresh weight (Kawashiro et al., 2008). It is assumed that this substance is able to cross the placental barrier and affect the fetus.

The degradation of TBP has been studied in several experiments. Contreras et al. (2009) reported the removal up to 92% of the substance *via* a catechol-driven Fenton reaction under appropriate conditions. When studying the biodegradation of TBP by a consortium of soil microorganisms, the reported half-lives were 8–10 days for aerobic conditions and 7 days for anaerobic conditions (Nyholm et al., 2010). Cultures of microorganisms were also tested from the sludge of wastewater treatment plants after various treatment procedures (activation, digestion, and sanitation). These results showed that the activation process for wastewater treatment plants enhanced TBP degradation, which resulted in the accumulation of bromides (Brenner et al., 2006). TBP aerobic degradation by bacterial communities from psychrophilic lakes is reported in another study (Aguayo et al., 2009). TBP biodegradation by ligninolytic fungi has also been studied. Donoso et al. (2008) showed that a strain of the basidiomycete *Trametes versicolor* isolated from a Chilean forest not only has high resistance against this compound (maximum tolerated concentration 80 μg/ml), but could also rapidly degrade it. On day 4 of cultivation, 98.5% of the original TBP concentration (20 mg/L) was removed and the whole biodegradation process was accompanied by stimulation of the activity of phenoloxidase (*i.e.* laccase), an enzyme that is active in the breakdown of phenolic compounds. In a similar study, slightly slower TBP degradation kinetics were observed using another strain of *T. versicolor*; the removal after 4 days of incubation was about 65% of the original amount added (1 mM) (Uhnaková et al., 2009).

In conclusion, TBP is a ubiquitous, well-known substance that is currently used in a number of applications, including use as a flame retardant, and it is widely distributed in the environment. Biodegradation of this substance is relatively rapid under various conditions; however, it must be borne in mind that it could have a major impact on humans, especially on the processes of hormonal regulation. The necessary legislation to ban this substance is currently lacking.

3.10. Decabromobiphenyl (BB209)

BB209 belongs to the group of brominated biphenyls. Use of this substance began in the 1970s as a commercial mixture, initially containing lower brominated congeners. However, accidental contamination of animal feed with hexabromobiphenyl in Michigan led to a ban on hexabromobiphenyl flame retardants in the USA in 1974 (WHO, 1994). Nevertheless, the production of octaBB and decaBB formulations continued until 1979. It has been reported that brominated biphenyls were produced in Europe until the year 2000 (Alaee et al., 2003). Commercial mixtures now available have different names depending on the manufacturer, such as Berkflam B 10 or Flammex B-10, produced in London (WHO, 1994). Polybrominated biphenyls are used as flame retardants in textiles, electronic equipment and plastics (Vetter et al., 2008), and are also used in ABS (Hirschler and Tsika, 1983; Donaldson et al., 1983). According to the European Union regulations, the use of BB209 in products is tolerated up to a content of 0.1% by weight (European Commission 2005, European Commission Decision 2005/618/EC).

BB209 volatilization from ABS was reported at elevated temperatures (Donaldson et al., 1983) and it is therefore probably present in indoor air. This retardant was detected in dust collected in an electrical waste storage plant, where BB209 reached an average concentration of 260 ng/g (Muenhor et al., 2010). Other authors also reported the presence of BB209 in the dust collected near a pond system at a concentration of 37.9 ng/g per dry weight (Sawal et al., 2008). It is assumed that dehalogenation of BB209 occurs in the environment or in animal tissues, resulting in the formation of less brominated congeners (Vetter et al., 2008).

Several studies demonstrated the ability of BB209 to activate the AhR receptor in pig liver cells. This activation increased with an increase in the BB209 concentration up to the highest concentration investigated (20 μM) (Alonso et al., 2008). From the structural (steric) point of view, this substance is incapable of fitting into the receptor binding site, raising further questions about the possible mechanisms of AhR activation (Alonso et al., 2008). Brown et al. (2004) confirmed the ability of BB209 to activate AhR under *in vitro* conditions.

It is rather surprising that BB209 has not been investigated in depth. Little is known about BB209 degradation in the various environmental compartments. Due to its fully-brominated character, the only known and theoretically likely possible mechanism is anaerobic dehalogenation (Field and Sierra-Alvarez, 2008; Furukawa and Fujihara, 2008). However, to the best of our knowledge, the halorespiration mechanism has not yet been described for BB209.

3.11. Tetrabromobisphenol A bis-(dibromopropyl ether) (TBBPA-BDBPE)

This novel fire retardant, along with other substances such as tetrabrombisphenol A-diallyl ether, represents a variant of tetrabromobisphenol A. TBBPA-BDBPE is manufactured under various brand names such as SAYTEX HP-800A or PE-68 and is used as a flame retardant in HIPS, polypropylene and other polymers. The production of this substance in 2006 was 4000 t in the People's Republic of China and 4500 t in the USA (Covaci et al., 2011).

DBPE-TBBPA is not acutely toxic. LD_{50} in mice was found to be greater than 20 g/kg for oral and dermal administration. No abnormal gross symptoms or death were observed (WHO, 1995). The induction of cytochrome P450 1A1 was not observed under *in vitro* conditions (Pullen et al., 2003). Mutagenicity tests performed with *Salmonella typhimurium* strains TA100 and TA1535 (Haneke, 2002b) yielded positive responses both in the presence and in the absence of metabolic activation, pointing to the mutagenic activity of this substance. In addition, the mutagenicity in the absence of metabolic activation was assessed in strain TA98, suggesting that metabolism promotes the formation of less mutagenic substances (Haneke, 2002b). No endocrine effects of TBBPA-BDBPE were found in tests based on aromatase enzymes (CYP19 and CYP17) in H295R adrenal cortical cells (Canton et al., 2005). Furthermore, no effects of TBBPA-BDBPE on AhR, the androgen receptor, progesterone receptor and estrogen receptor were recorded. On the other hand, TBBPA-BDBPE has a high ability to inhibit estradiol sulfotransferase and can compete with thyroxine for binding sites for the protein transthyretin. This ability is similar, although lower, than that reported for tetrabromobisphenol A (Hamers et al., 2006).

In relation to the effects on humans, only one case of skin and eye irritation was reported, when three workers were exposed to

TBBPA-BDBPE-containing thermoplastic resins (Haneke, 2002b). To date, however, only a few scientific articles have dealt with the occurrence of this compound in the environment and its biodegradability. TBBPA-BDBPE was detected in seepage water (average concentration 81 ng/L) from a metal recycling factory, but not in the sediment from the same location originating in Norway (Nyholm et al., 2013). TBBPA-BDBPE has also been found in herring gull eggs sampled in North America (Letcher and Chu, 2010) and dust collected near an artificial stream and pond system in Berlin, Germany (Sawal et al., 2008).

Studies on the metabolism of this substance in rats (Fischer 344) showed that TBBPA-BDBPE is not metabolically transformed. It was also found that, following oral administration, only a minor quantity of this substance was absorbed in the body, with absorption half-life of 2.5 h. On the other hand, elimination from the body took a relatively long time (13.9 h) (Knudsen et al., 2007) and most of the retained compound accumulated in the liver.

TBBPA-BDBPE has been detected in various environmental samples (Sawal et al., 2008; Shi et al., 2009). It has been reported that TBBPA-BDBPE may not be readily biodegradable (WHO, 1995; Rahm et al., 2005). Rahm et al. (2005) reported that this substance is susceptible to dehydrohalogenation at the same rate as DDT.

4. Conclusions and suggestions for further research

The information summarized above clearly demonstrates that several NBFR that replaced restricted compounds have frequently been detected in various compartments of the environment. This provides evidence for their recalcitrance and tendency to persist under natural conditions. Most of these properties can be generally linked to their low chemical reactivity, high hydrophobicity and presence of bromine atoms, which hamper their decomposition *via* biotransformation processes. This evidence is further supported by the documented bioaccumulation potential of some BFRs and NBFRs in animal tissues. BFRs and NBFRs are mixed into plastics and foams but often do not form chemical bonds; after release from these matrices, a number of compounds bind to aerosol particles and therefore they have been recognized as significant pollutants of the indoor air environment. However, numerous studies demonstrated that BFRs can be detected in the environment far from the locations where they are produced and/or used, and that these concentrations are steadily increasing, both in the environment and in humans. This is probably also due to their high production volumes, widespread usage, and the mentioned environmental persistence. Thus BFRs and NBFRs have become ubiquitous contaminants in the environment.

Surprisingly, little toxicity information is available for many existing BFRs and NBFRs. In the EU, the requirements are defined by REACH. This testing includes very basic acute, sub-chronic or chronic tests; however, specific tests for instance endocrine disruptions are not tested. Additionally the basic toxicity and biodegradability testing is required for chemical compounds that are produced in the EU. Generally, low acute toxicity values have been reported for the BFRs and NBFRs tested using model organisms; however, a few studies addressing long-term (chronic) effects and endocrine disrupting activity provided evidence for (anti-) estrogenic and (anti-)androgenic features, thyroid function disruption, immunotoxicity, behavioral modification and gastrointestinal lesions. Consequently, organobromines might pose much higher risks to both humans and wildlife than so far anticipated.

The present review article highlights the lack of knowledge concerning NBFR biotransformation processes and the mechanisms by which these compounds prompt ecotoxicological or hormone-like responses. Considering their negative effects during accidents involving fires, their useful properties are questionable. The results reviewed in this paper also emphasize the need for further research regarding their fate in the environment, ecotoxicological properties and technologies leading to their decomposition, in order to prevent undesirable release into the environment, mainly from industrial processes.

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Photocatalytic water treatment on TiO_2 thin layers

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ABSTRACT

Photocatalysis is generally applied as a suitable technique for water decontamination and/or purification, especially for decomposition of endocrine disruptors. Endocrine disruptors are commonly present not only in wastewater but also in natural water. Endocrine disruptors are persistent to degradation by common chemicals as well as biological and photolytic processes. Decomposition of three representative endocrine disruptors (17-ethynyl estradiol, bisphenol A, and 4-nonylphenol) was tested on previously prepared TiO_2 photocatalyst in two types of reactors; a batch reactor and a plug-flow reactor. TiO_2 thin layers deposited on three various substrates were prepared by a sol–gel process with employment of a dip-coating technique for subsequent application. Properties of the prepared layers were thoroughly characterized by XRD, SEM, AFM, UV–vis, and Raman spectroscopy. Photo-electrochemical properties were determined by linear voltammetry and amperometry to obtain photoinduced properties of the prepared TiO_2 photocatalyst which corresponded to the photocatalytic activity. Photocatalytic decomposition efficiency was evaluated with respect to individual compounds for both reactors together with values of toxicity and estrogenic activity during the photocatalytic decomposition process. Furthermore, resistance of individual compounds to the photocatalytic decomposition process was evaluated together with possible formation of intermediates or by-products.

Keywords: Water purification; Photocatalysis; Endocrine disruptor; TiO_2 layers

1. Introduction

Environmental pollution is currently becoming an increasingly serious problem of our time. The rising amount of emerging group of environmental pollutants in wastewater, known as endocrine disrupting compounds (EDCs), starts to be alarming. EDCs are natural and synthetic chemicals having adverse effects on human beings and animals via influencing

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endocrine systems [1]. Due to their influence on hormonal balance of multicellular organisms, EDCs constitute a real threat in wastewater. Among EDCs, 17b-ethynylestradiol (EE2) excreted by animals and human beings at ng L^{-1} levels in surface waters was identified to have the highest endocrine disrupting activity. Similarly to EE2, other EDCs also resist degradation of typical sewage treatment operations and they are released into surface waters. With a particular concern for human health, EDCs must be treated before entering public drinking water systems [2].

An intensive effort has been focused on removal of single EDCs or their mixtures which belong to the group of environmental pollutants from polluted water by TiO_2 photocatalysis. Owing to that, conventional methods of water and sewage treatment are not completely effective in removing the EDCs; advanced oxidation processes have been intensively studied as suitable technologies for EDCs degradation [3–8].

TiO_2 in powder or a thin layer form has been a promising semiconductor widely used in the field of water or air purification. It is well established that titanium dioxide (TiO_2) in the presence of UV light can create very active species that are able to restore and preserve the environment clean by decomposition of harmful organics. TiO_2 has awakened a great interest due to its chemical stability, low toxicity, low cost, photoinduced ability, and other specific properties. It can be prepared as powder, in a thin layer form, fibers, or tubs [9–12]. TiO_2 is a *N*-type semiconductor due to oxygen vacancies and its conductivity is caused by the absorption of light with corresponding energy which evokes generation of charge carrier species (electron–hole pairs). The anatase structure presents excellent photoinduced properties caused by the ability of OH radical creation on the anatase surface. TiO_2 absorbs only in the UV area due to the width of energy band gap (3.20 eV for anatase, 3.02 eV for rutile); therefore, the UV lamp for activation has to be used [13]. This fact can greatly limit utilization of TiO_2 in some applications.

This metal oxide was initially used as white pigment utilized in paint, plastic, ceramics, glass, and paper production [14–16]. TiO_2 pigment is also used in food industry (dye E171) and in pharmaceutical industry (tooth paste, drugs, sunbathing cream). The main field of TiO_2 application is catalysis and photocatalysis. This oxide used to be applied as promotor, support, or catalyst (Fisher–Tropsch synthesis [17,18]). Concerning photocatalysis, it is used in selective reduction of NOx to N_2, VOC degradation, photocatalytical air or water purification [19–24]. Its antibacterial properties can be applied for destruction of biological organisms (self-cleaning application). It is important to note that these cleaning and antibacterial functions of TiO_2 are obtained without using any chemicals, it needs only light. This metal oxide has found its use in various applications and new ones are being created.

This article deals with water treatment (removal of endocrine disruptors) by application of TiO_2 thin layers. Photocatalysis on TiO_2 seems to be a very promising technique for solving these environmental pollution problems. For these reasons, immobilized TiO_2 was applied for EDCs degradation. Degradation efficiency on TiO_2 thin layers was studied in batch and plug-flow reactors together with evaluation of toxicity and estrogenity of reaction products.

2. Experimental

2.1. Thin layer preparation

The templated sol–gel process was based on hydrolysis followed by polycondensation of metal precursor in organic matrix. The homogenous sol was prepared by addition of titanium alkoxide (TTIP, 99.999%, Sigma-Aldrich) used as Ti(IV) precursor into the inverse micellar solution. Reverse micelles were created by molecular templates Triton X 114 (non-ionic surfactant, Sigma-Aldrich) in the nonpolar environment of cyclohexane (Aldrich, 99.9%, HPLC grade). Sol–gel reactions took place in the core of a reverse micelle containing a small amount of water. The sol application was performed by a laboratory dip-coater (ID-Lab coater 4). Three supports were used for TiO_2 films deposition; glass beads with diameter 1.5 mm, glass tubes with inner diameter 4.5 mm, or indium tin oxide conductive glass (ITO, 5–15 X, Delta-Technologies Ltd). Three cycles of the dip-coating method with velocity 6 cm min^{-1} were applied. Among the single dip-coating cycles, samples were thermally treated by calcination at 450°C for 4 h with the temperature ramp 1°C min^{-1} during air flow in a muffle furnace. The same sol and treatment were also used for TiO_2 preparation in a powder form used for determination of TiO_2 texture characteristics.

2.2. Layer's characterizations

A crystallographic structure of prepared thin films was performed by X-ray diffraction (Panalytical-MRD laboratory diffractometer with the Cu anode) and Raman spectroscopy (Raman Dispersive Spectrometer Nicolet Almega XR, with wavelength 473 nm). Surface morphology was studied by a scanning electron microscope (Hitachi S4700) and AFM microscope (Thericroscopes). Layer thickness on the glass

substrate was evaluated from the pictures made by SEM where the layer edge was depicted. Values of absorption edges of layers deposited on glass were obtained from UV–vis spectra (PerkinElmer Lambda 35 equipped with a Labsphere RSA-PE-20 integration sphere).

Textural properties of the prepared photocatalyst in a powder form were evaluated from N_2 physisorption performed on the volumetric instrument ASAP 2020 (Micromeritics, USA). Before the analysis, samples were dried at 105°C for 24 h at 0.1 Pa. Adsorption–desorption isotherms were measured by nitrogen at 77 K. The specific surface area (S_{BET}) was evaluated by the BET method; micropore volume and the mesopore surface area (S_{meso}) by the *t*-plot method with Lecloux–Pirard master isotherm; and pore-size distribution by the advanced BJH method [25].

2.3. Photocatalytic experiments

Two types of reactors, batch and plug-flow, were used for photocatalytic experiments. A batch reactor possesses the volume of reaction mixtures of 200 mL with 2.5 mg of TiO_2 supported on 5 g of beads. Experiments were performed under continuous shaking for 7 h. Four tubes with the length of 10 cm and inner diameter of 4.5 mm were applied as a plug-flow reactor with the volume 6.36 cm^3. 2.2 mg of TiO_2 covered the inner surface of tubes. Applied flow rates varied between 0.2 and 2 $cm^3\ min^{-1}$.

Three endocrine disruptors, EE2, bisphenol A (BPA), and 4-nonylphenol (4NP) with initial concentration of 10 ppm, were used for photocatalytic degradation tests in both reactors. For photocatalyst activation a UV lamp (Philips HOK 4/120 SE, 400 W medium pressure mercury lamp) with the wavelength of 250–420 nm was employed in the 20 cm-distance. Lamp intensities for individual UV light regions are shown in Table 1.

2.4. EDC, toxicity, and estrogenity analyses

EDC analyses were performed on Waters Alliance HPLC module equipped with a PDA detector, column thermostat, and Empower software. Identification was based on standard retention time and UV spectra by consensus (maximum 280 nm). The toxicity test is standardized as determination of the bioluminiscence inhibition of *Vibrio fischeri* bacteria strain. For this purpose, a gram negative bacteria strain *V. fischeri* NRLL-B-11177 was used as a testing microorganism. Bacteria emit the light arising in the organism during the chemical reaction catalyzed with enzyme luciferase. The visible light was reduced at the contact of bacteria with the contaminant. Bioluminescence reduction was measured in 15 and 30 min intervals by a luminometer.

Estrogenity was tested on a *Saccharomyces cerevisiae* sp. strain with an integrated receptor for human estrogen and androgen compounds. Yeast cells in contact with hormonal active agents produce luciferase, which creates light measured by a luminometer, thanks to luciferin present in the medium.

Table 1
Lamp intensity for individual UV light regions

Type of light region	Intensity ($W\ m^{-2}$)
UV–vis	69
UVA	49
UVB	11
UVC	1

3. Results and discussion

3.1. Structural properties

Prepared photocatalysts in a thin layer form deposited by the dip-coating method on various substrates and calcined at 450°C possess the crystallographic structure of anatase. The crystalline form of the pure anatase was detected by data evaluation from XRD analyses and was confirmed by Raman spectroscopy. In Fig. 1(a), the main diffraction lines of anatase are depicted. Raman spectra (Fig. 1(b)) show attendance of characteristic vibration lines with 640, 525, 400, and 150 cm^{-1}, which confirms the pure anatase structure.

The layer morphology was studied by SEM and by AFM analyses (see Fig. 2(a) and (b)). The detailed view of the film surface made by SEM shows a smooth porous surface without any higher extent of surface defects. This fact is corroborated the results obtained by AFM analyses. Relative surface roughness expressed as an RMS factor (roots mean square values) is lower than 1, which indicates a very smooth surface of prepared layers.

Film thickness was evaluated from SEM images depicting the layer edge (Fig. 3). The thickness of 3 deposited layers was approximately 330 ± 10 nm. Moreover, clusters of TiO_2 particles were also easily detectable. Their size was about 20 nm and the particle size, which was evaluated from XRD diffraction lines, was 7 ± 1 nm. The absorption edge of prepared thin layers was determined from absorption spectra approximately at 355 nm. The prepared photocatalyst possessed a S_{BET} of about 82 $m^2\ g^{-1}$ formed mainly by the mesopore surface (S_{meso} = 62 $m^2\ g^{-1}$) with porosity of 48%.

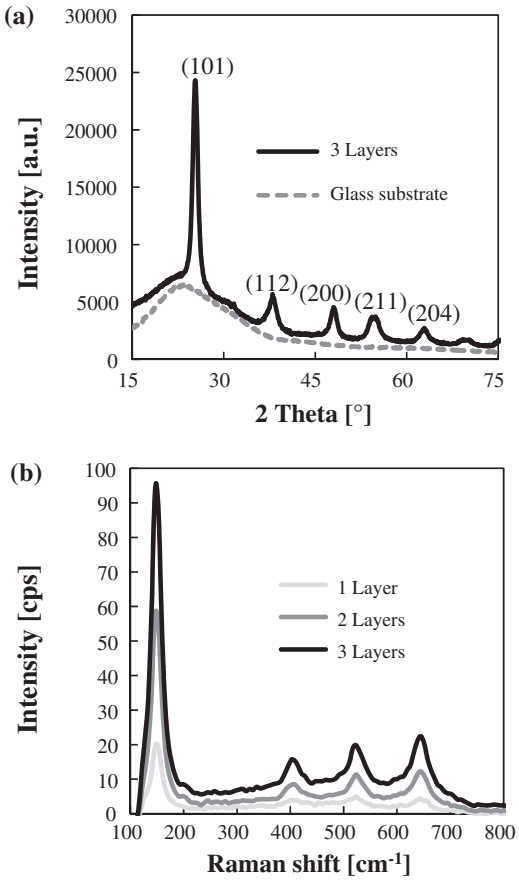

Fig. 1. XRD patterns (a), Raman spectrum (b).

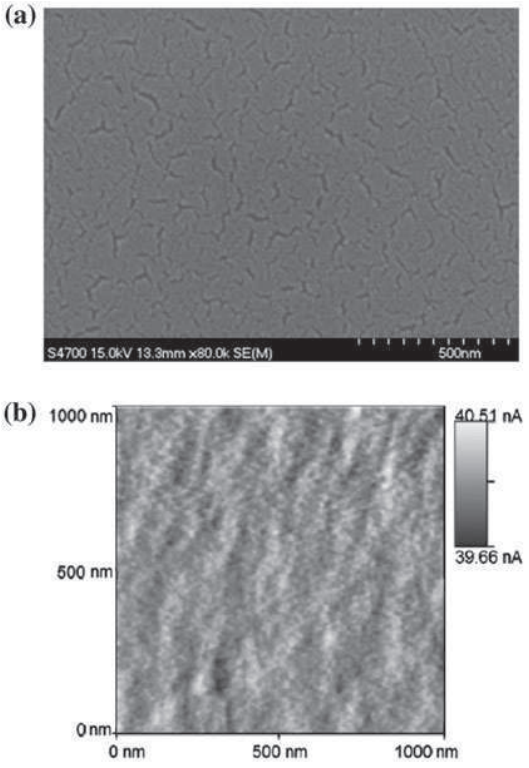

Fig. 2. Layer surface made by SEM (a), AFM analyses of the layer morphology (b).

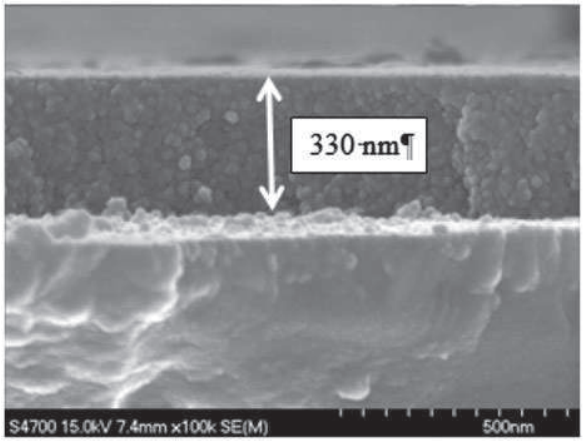

Fig. 3. Layer thickness.

3.2. Photocatalytic experiments

Degradation of EDCs in water solution was studied on prepared TiO_2 layers in two types of reactors; a batch reactor and a plug-flow reactor. Three representative endocrine disruptors were selected for testing: EE2, which represents a pharmaceutical substance of hormonal contraception; BPA, which is released e.g., from food packages to water; and 4-NPh, which belongs to biodegradation products of detergents and antioxidants. It is necessary to emphasize that only photocatalysis (no photolysis) takes part in degradation processes. Lamp intensities for individual UV light regions are obvious from Table 1. Obtained results for all three EDCs in the bath reactor during 7 h experiments are depicted in Fig. 4. It is evident that total decomposition of EE2 was achieved even after 5 h with excellent reproducibility. 4-NPh and BPA revealed higher resistance to the photocatalytic degradation process compared with EE2. 30% of 4-NPh and only 10% of BPA were decomposed after 7 h. Nevertheless, reproducibility of 4-NPh and BPA experiments corresponded to EE2 reproducibility with standard deviation (SD) lower than 3%.

During photocatalytic decomposition experiments, some new intermediates or by-products are usually created and these compounds can possess higher estrogenity and toxicity than the original contaminants. Therefore, estrogenity and toxicity of EDC

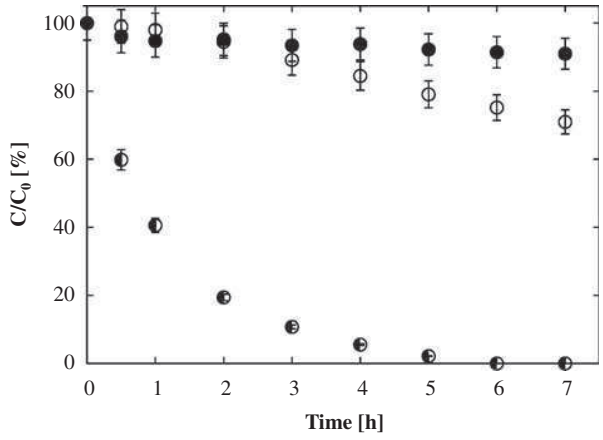

Fig. 4. Decomposition course of 4-NPh ◐, EE2 ○, and BPA ● in batch reactor.

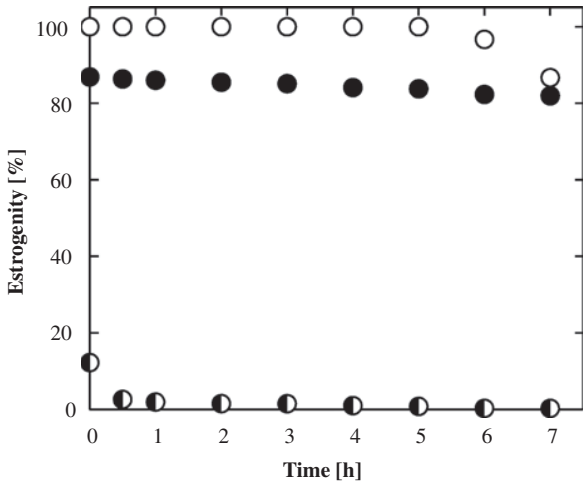

Fig. 5. Estrogenity course of 4-NPh ◐, EE2 ○, and BPA ● in batch reactor.

decomposition were determined. Obtained data for all three contaminants are summarized in Figs. 5 and 6. It is obvious that efficiency of photocatalytic process on estrogenity and toxicity values significantly varied for three tested compounds. Residual estrogenity decreased in the range of EE2, BPA, 4-NPh and toxicity in the range of BPA, 4-NPh, EE2. However, both estrogenity and toxicity of all compounds decreased during experiments. Thus, no emerging products, intermediates, or by-products revealing higher estrogenic activity and toxicity than original compounds were created.

It can be summarized that the slow gradual decrease of BPA estrogenity and toxicity corresponds to its decomposition rate. On the contrary, 4-NPh

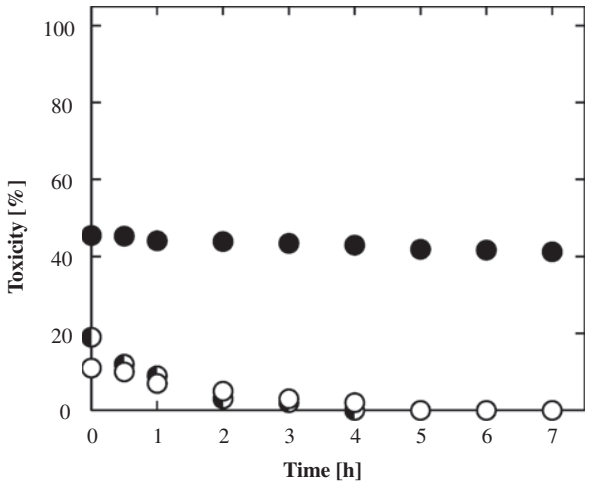

Fig. 6. Toxicity course of 4-NPh ◐, EE2 ○, and BPA ● in batch reactor.

revealed no estrogenity and toxicity after 2 h similarly to EE2 toxicity. However, values of EE2 estrogenic activity decreased slowly (10% after 7 h) with respect to quick decomposition. This fact points to the formation of more stable degradation intermediates, whose decomposition is slower.

The photocatalytic activity of TiO_2 layers deposited on an inner surface of plug-flow reactor tubes was also tested on decomposition of EE2, BPA, and 4-NPh in water solution. Obtained results for two various flow rates, 0.5 and 0.2 $cm^3\ min^{-1}$, are shown in Fig. 7. It is evident that decomposition of individual compounds corresponds to the data obtained in a batch reactor and depends on retention time. The best results were obtained for 4-NPh. At flow rates 0.5,

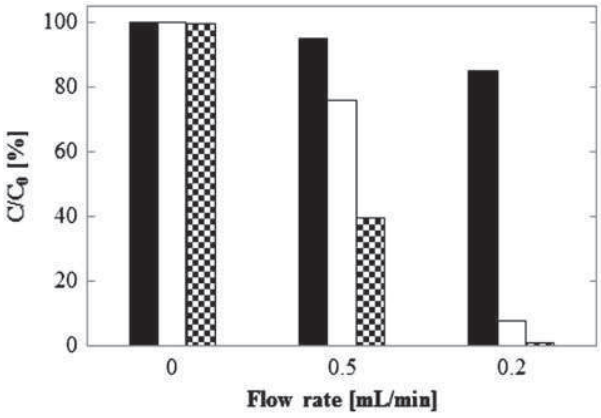

Fig. 7. Decomposition of ■ BPA, □ EE2, ⊡ 4-NPh at flow rates 0.5 and 0.2 $cm^3\ min^{-1}$.

Table 2
Decomposition of 4-NPh at flow rate 0.5 $cm^3\ min^{-1}$

Experiment	1 (mg/L)	2 (mg/L)	3 (mg/L)	4 (mg/L)	$C_{average}$ (mg/L)	D_{max} (%)	SD (mg/L)
C_{output}	4.928	4.768	4.826	4.850	4.843	1.75	0.0663

60% efficiency and at 0.2 $cm^3\ min^{-1}$ even 99% efficiency of degradation process was achieved. Slightly worse results were obtained for EE2. Efficiency at flow rates 0.5 and 0.2 $cm^3\ min^{-1}$ was 24 and 92%. The most resistant compound was again BPA with maximal 15% efficiency of the photocatalytic process. Photocatalytic degradation of all compounds for individual flow rates was measured repeatedly to guarantee reproducibility of experiments. Obtained results for 4-NPh and flow rate 0.5 $cm^3\ min^{-1}$ are summarized in Table 2. It is evident that reproducibility of results is excellent with maximal deviation (D_{max}) 1.75% and SD 1.3%.

Decomposition of EE2 for four different flow rates is shown in Fig. 8, where obtained conversions on retention time are depicted. It is obvious that linear dependence was obtained with the correlation coefficient of 0.99. Thus, in agreement with literature [26], the first reaction order was evaluated.

Estrogenity and toxicity values of individual compounds decrease during the decomposition process and correspond to the values obtained for the batch reactor. Results for 4-NPh at flow rate 0.5 $cm^3\ min^{-1}$ are shown in Fig. 9, where the vertical line stands for retention time of the reactor 12.8 min. It can be seen that during the photocatalytic degradation experiment, values of toxicity and estrogenic activity copied the decreasing trend of 4-NPh concentration; which means that they corresponded to the degradation process.

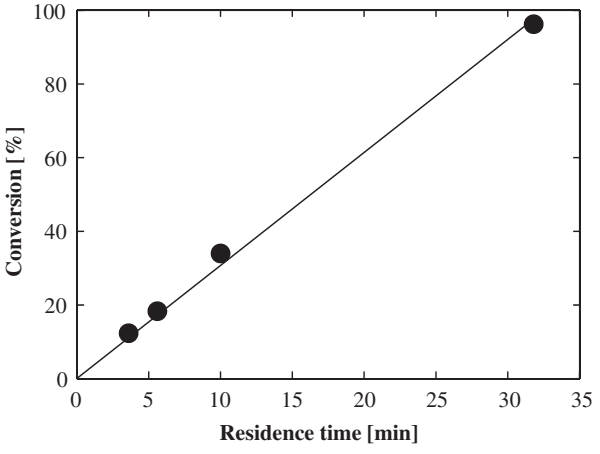

Fig. 8. Dependence of EE2 conversion on residence time for EE2, points—experimental, line—calculated ($y = 3.0712x$, $R^2 = 0.9965$).

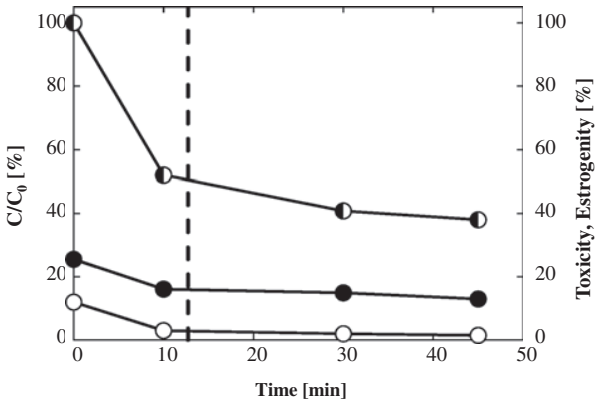

Fig. 9. Dependence of 4-NPh concentration ◐, 4-NPh toxicity ●, and 4-NPh estrogenity ○ on time, retention time of reactor.

4. Conclusions

Decomposition of three representative endocrine disruptors (EE2, BPA, and 4-NPh) on TiO_2 layers as a photocatalyst was tested in two types of reactors, a batch reactor and a plug-flow reactor. The glass beads covered by a thin layer of TiO_2 were applied as a photocatalyst in the batch reactor. In the plug-flow reactor, the thin layer of TiO_2 was supported on the inner reactor walls.

Based on minimally three times repeated experiments, it was verified that the total decomposition of 4-NPh was achieved after 5 h. However, 4-NPh and BPA revealed higher resistance to the photocatalytic degradation process compared with EE2. 30% of 4-NPh and even only 10% of BPA were decomposed after 7 h. Nevertheless, higher efficiency was obtained for the plug-flow reactor with TiO_2 photocatalyst deposited on the inner surface of tubes. The degradation process in plug-flow reactor provided even 98% efficiency to 4-NPh and 95% efficiency to EE2 at flow rates 0.2 $cm^3\ min^{-1}$. Similarly to the batch reactor, the most resistant compound was again BPA with maximal 15% efficiency of the photocatalytic process at the same flow rate. It is necessary to stress that the

excellent reproducibility was achieved for all experiments in both reactors.

Values of toxicity and estrogenic activities during the photocatalytic decomposition process significantly varied for three tested EDC without any influence of the reactor type. The slow gradual decrease of BPA estrogenity and toxicity corresponds to its decomposition rate and corroborates its high resistance to the photocatalytic decomposition process. On the contrary, the estrogenic activity and toxicity of 4-NPh rapidly decreased similarly to its concentration. The most interesting are the values of EE2 estrogenic activity. Estrogenity decreased really slowly regarding the quick decomposition of EE2. This fact points to the formation of more stable degradation intermediates. However, both estrogenity and toxicity of all compounds substantially decreased during experiments; thus, no emerging products, intermediates, or by-products revealing any higher estrogenic activity or toxicity than original compounds were created during the decomposition process.

Based on these results, it can be summarized that photocatalytic decomposition of endocrine disruptors on TiO_2 layers, especially in a plug-flow reactor, seems to be an efficient degradation method and a promising technique for final water purification as the last part of sewage treatment plant.

Acknowledgment

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Endocrine disruptor degradation by photocatalytic pilot plant unit

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***Abstract*— Degradation of endocrine disruptors on an immobilized titanium dioxide photocatalyst in the presence of UV light was tested on a specially designed plug-flow reactor. The active part of this pilot plant reactor with the inner free volume of 3.5 L consisted of stainless steel annulets coated by titanium dioxide thin layers in a zig-zag arrangement. Two representative endocrine disruptors, 17-ethynyl estradiol and bisphenol A, in water solution were chosen for testing of the designed pilot plant reactor. Efficiency of the reactor was evaluated for various concentrations and flow rates. Process effectiveness and high reproducibility was also confirmed by estrogenity and toxicity tests. The designed photocatalytic treatment system was tested by purification of real waste water containing seven main endocrine disruptors and rinsed water from pharmaceutical industry. Efficiency of the photocatalytic degradation process varied between 44 – 100 % with respect to the individual endocrine disruptors and flow rates. Possible utilization of the photocatalytic reactor as the last purification unit at sewage plants was confirmed even for flow rates up to 300 L/h.**

Keywords — Endocrine disruptor; Titanium dioxide; Photocatalysis; Waste water treatment; Pilot plant photo-reactor

I. INTRODUCTION

Endocrine disruptors (EDCs) have been receiving a growing worldwide attention owing to their potential negative impacts on human health and environment. Endocrine disruptors belong to exogenous hormonally active agents influencing the hormonal system of animals and humans and can negatively affect their development. These compounds possess a very large chemical diversity, which influences their different endocrine activities. Agency for Environmental Protection (EPA) has established a list of potential substances with endocrine activity of over 10,000 molecules [1]. One of the first study focused on the EDCs is the publication written by T. Colborn (1993) [2]. The ability of EDCs to bind to steroid hormone receptors led scientists to a closer investigation of the endocrine disruptor hazard, particularly on wildlife and humans [3-6]. Moreover, endocrine disruptors may be hormonally active in very low concentrations and they may cause chronic disorders during a long-term exposure [7].

Regarding the fact that conventional methods of water and sewage treatment are not completely effective in removing the EDCs, Advanced Oxidation Processes (AOPs) have been intensively studied as really promising technologies for EDCs degradation [8-13]. Especially, heterogeneous photocatalysis has evoked a great interest in treatment of various types of organic contaminants found in polluted water or air. This method is based on the formation of highly reactive species which can degrade even the most recalcitrant molecules. The most commonly used photocatalyst is titanium dioxide (TiO_2) applied in the form of powder or immobilized as a thin layer. An intensive effort has been focused on removing single endocrine disrupting compounds (EDCs) or their mixtures which belong to the group of environmental pollutants from polluted water by TiO_2 photocatalysis.

Nevertheless, most studies were usually carried out in a laboratory scale [14-20], and studies concerning pilot scale units are rare. Moreover, the total majority of pilot plant experiments combine the TiO_2 photocatalysis with other AOPs such as Fenton or photo-Fenton treatment, the solar UV-light system or the addition of hydrogen peroxide [20-22] together with membrane processes such as cross-flow microfiltration to guarantee the TiO_2 powder removal [23]. Other pilot studies were limited to experiments with solar type pilot plants [24-27]. Thus, there is a lack of literature dealing with the application of TiO_2 photocatalysis in the pilot plant scale. Moreover, this method does not indicate the formation of estrogenically active transformation products during the treatment and, therefore, it seems to be highly effective and proficient for water purification.

Consequently, this study is focused on water treatment containing various EDCs by application of photocatalytic oxidation in a specially designed pilot plant unit with immobilized TiO_2. Efficiency of this reactor was tested not only on simulated polluted

water but also on real waste water after the sewage plant treatment or real water from pharmaceutical industry with special focus on representative EDCs such as 17b-ethynyl estradiol, bisphenol A, norethisteron, danazol *etc*.

II. Materials and Method

A. Preparation of TiO_2 layer

TiO_2 thin layers were prepared by a sol-gel process controlled in a reverse micellar environment by a dip-coating technique. TiO_2 was synthesized by the addition of Titanium (IV) isopropoxide ($Ti(OCH(CH_3)_2)_4$, Aldrich, > 97%) into the formed inverse micellar solution made of cyclohexane (Aldrich, 99.9+%, HPLC grade), non-ionic surfactant Triton X114 ($C_{27}H_{48}O_{7.5}$, Aldrich) and distilled water. The molar ratio of cyclohexane/Triton X114/water/$Ti(OC_3H_7)_4$ was kept at 11/1/1/1 (volume ratio TX114/cyclohexane = 0.49) [28]. Thin TiO_2 films were deposited by four cycles of a dip-coating method. The substrate was dipped into the sol during 30 s and then it was pulled out with the velocity of 6 cm min^{-1}. At the time between the single dip-coating cycles, the samples were thermally treated by calcination at 450 °C for 4 h with the temperature ramp 1 °C min^{-1} in an air flow in a muffle furnace. As a substrate, 21 stainless steel annulets of 2 different averages were used. The larger annulet had 240.5 cm^2, and the smaller one 224.2 cm^2.

B. Layer characterization

Structural analyses of the prepared thin TiO_2 films were performed by X-ray diffraction (Panalytical-MRD laboratory diffractometer with the Cu anode), Raman spectroscopy (Raman Dispersive Spectrometer Nicolet Almega XR, 473 nm laser), scanning electron microscopy (Hitachi S4700) and atomic force microscopy (Thericroscopes). The layer resistance was measured by an abrasion tester Elcometer 1720.

C. Decomposition of endocrine disruptors

17α-ethynylestradiol (EE2; Sigma–Aldrich, ≥ 98.0 % HPLC) and bisphenol A (BPA; Sigma–Aldrich, ≥ 99.0 %) were chosen as the representative endocrine disruptors for tests with simulated water. The initial applied concentration of EE2 and BPA was 10 ppm. The average initial concentrations of the monitored EDCs in the real waste water after the sewage plant treatment are summarized in Table I. for three main contaminants (bisphenol A (BPA), irgasan (IRG) and 4-nonyphenol (4-NPH)).

TABLE I. Endocrine disruptors in real waste water

Substance	**Concentration** [ng/L]
BPA	387.2
IRG	180.2
4-NPH	59.6

Decomposition of EDCs was studied by a photocatalytic process by means of the UV light activated TiO_2 thin layers. Irradiated TiO_2 creates active radicals which can react with molecules of water or directly with endocrine disruptors and form oxidative products and intermediates. This process can be described by a simple mechanism (Equation 1 – 4).

$$TiO_2 + hv \rightarrow TiO_2 (e^- + h^+) \quad (1)$$
$$h^+ + H_2O \rightarrow OH^{\bullet} + H^+ \quad (2)$$
$$e^- + O_2 \rightarrow O_2^{\bullet} \quad (3)$$
$$h^+/OH^{\bullet} + ED \rightarrow \text{Oxidative products} \quad (4)$$

The degradation of EDCs in water was evaluated on the basis of the concentration decrease. The analysis was performed on Waters Alliance HPLC module equipped with PDA detector, a column thermostat and Empower software. Standard retention time and UV spectra by consensus (maximum 280 nm) were used for identification.

Kinetic parameters of the reactions studied in photocatalytic system were determined by fitting experimental data to the theoretical model described by the equation (5), where, c_A represents the concentration of A in dependence on time, c_{A0} is the initial concentration of A, n represents experimental order of the reaction, k for reaction rate coefficient and τ for time.

$$c_A = c_{A0}[1+(n-1)c_{A0}{}^{n-1}k\tau]^{1/1-n} \quad (5)$$

The standardized determination of bioluminescence inhibition of *Vibrio fischeri* bacteria strain was applied [29] as a toxicity test. For this purpose, a gram of negative bacteria strain V. fischeri NRLL-B- 11177 was used as a testing microorganism. Bacteria emit the light arising in the organism during the chemical reaction and this visible light is reduced at the contact of bacteria with the contaminant.

Estrogenity was tested on a Saccharomyces cerevisiae sp. strain with an integrated receptor for human estrogen and androgen compounds. The yeast cells in contact with hormonal active agents produced luciferase, which together with luciferin present in the medium created light measured by luminometer.

D. Pilot plant experiments

According to the obtained data and knowledge based on the flow and batch reactors [30], a pilot reactor was built up. Photocatalytic experiments were performed in this special pilot plant reactor (Fig. 1) [31] placed at the sewage treatment plant area. The active part of the pilot plant reactor with the inner free volume of 3.5 L consisted of 21 stainless steel annulets coated by TiO_2 active thin layers. The reactor body uses a system of two tubs - an inner quartz tube and outer borosilicate glass tube. A medium pressure mercury lamp Philips HOK 25/120 with technical data: Overall Length C 367 mm; Diameter 21.6 mm; Lamp Wattage 2500 W; Lamp Voltage 440 V is situated inside the quartz tube. This UV lamp emits the polychromatic light beam which is cooled by air blowing in the inner tube and actuated by a ventilator.

The photocatalytic reactor is covered by a metal case to prevent UV light emission.

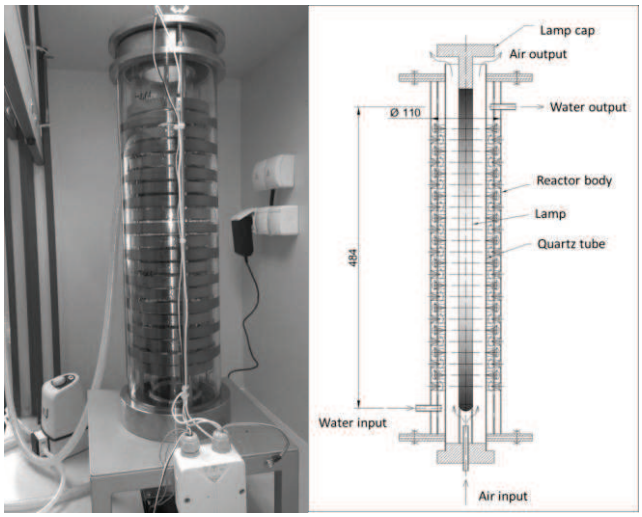

Fig. 1. *Pilot plant photocatalytic reactor*

The space between the inner tube and the outer tube is filled by rings with immobilized TiO_2 nanoparticles in a thin layer form. The contaminant water was pumped into the reactor at a constant rate and flow through the reactor annulus from the bottom upwards. The system of coated annulets was created by alternated organized bigger and smaller rings characterized by the shape of an isosceles triangle in a vertical sectional view (Fig. 2). This special arrangement provided the unique zigzag stream mode of contaminant water which ensured higher photocatalytic efficiency due to the increased UV irradiation transmittance and the larger irradiated area of the photocatalyst. The UV light emitted by the lamp homogenously irradiated the skew upper and lower area of the small and big rings, which caused photocatalysis and photolysis of organic compounds in the flowing contaminated water. The special zigzag water flow in the reactor led to a better transfer of the matter between molecules of contaminants and the photocatalyst surface, which also increased the photocatalytic activity.

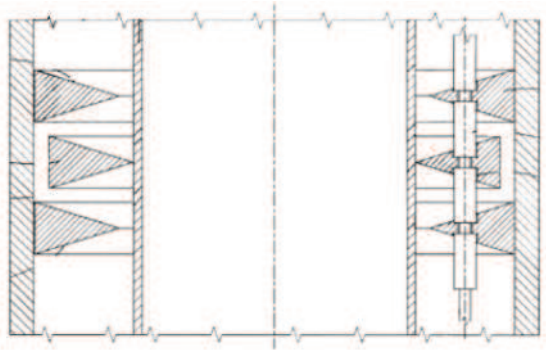

Fig. 2. *The coated annulets system with thin TiO_2 films*

III. RESULTS AND DISCUSSION

A. Layer's characterization

The prepared thin TiO_2 layers deposited on the metal rings possess the anatase crystalline structure, which was determined by XRD analysis and Raman spectroscopy. The thickness of three deposited layers was evaluated from images made by SEM and it was estimated at 330 nm. The layer surface morphology was studied by AFM analyses and the obtained value of RMS factor (< 1) indicated a smooth surface of layers. A more detailed characterization of layers was described in our previous work [32].

Regarding any application, layer resistance belongs to the most important properties. Therefore, the abrasion test was performed. A frictional element- felt disc with the density of 0.56 g cm-3 was applied as a tester. The abrasion resistance was tested by 1160 cycles with the weight of 0.25 kg and 2900 cycles with the weight of 0.5 kg. After these 4060 cycles, only 40-50 % of TiO_2 layers were removed, which corresponds to high abrasion resistance.

B. Degradation of simulated water

Two representative endocrine disruptors in water solutions were chosen for photocatalytic degradation in the designed pilot plant reactor with the volume of 3.5 L. EE2, which represents the pharmaceutical substance of hormonal contraception, and BPA, which is released e.g. from food packages, were selected as model EDCs. The efficiency of the degradation process was studied on simulated water with the initial concentration of both contaminants of 10 ppm. This model concentration of EDCs was chosen on the basis of the laboratory tests made in a flow photoreactor. The chosen concentration of EE2 similarly as BPA in simulated water was significantly higher than the usual sum of endocrine disruptors in real water at the output of sewage plant. Thus, the certainty of absolute purification of real water could be guaranteed even at high velocities.

Photocatalytic degradation of EE2 was measured for three various flow rates - 6, 9 and 14 L/h with corresponding retention time of 35, 24 and 15 min. The reaction course of EE2 decomposition during photocatalytic reaction in the reactor is depicted in Fig. 3. The efficiency of EE2 degradation process at flow rates 6 and 9 L/h achieved 95 % and 90 % respectively, while at 14 L/h the decomposition efficiency decreased to 78 %. The difference in efficiency between the flow rate 6 and 14 L/h is almost 20 % and it is evident that the degradation efficiency strongly depends on a used flow rate. In fact, the most important quantity is the retention time; a higher retention time causes a longer time of photocatalytic reaction of the solution on the TiO_2 layer surface and thus higher degradation efficiency.

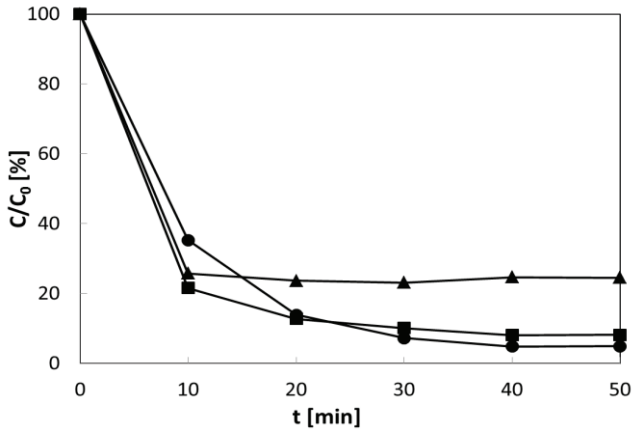

Fig. 3. *Decomposition of EE2 from model water at various flow rates, ● – 6 L/h, ■ – 9 L/h, ▲ – 14 L/h*

The photocatalytic degradation of BPA at three various flow rates - 5, 11 and 14 L/h is depicted in Fig. 4. The retention time of the solution during irradiation in the reactor was 45, 19 and 15 min, respectively. The highest conversion was reached at flow rate 5 L/h and the efficiency was 80 %. It can be seen that BPA is a more resistant compound to the photocatalytic process than EE2. Comparing the reached results at flow rate 14 L/h after 1 reaction cycle (15 min), it is obvious that degradation efficiency of EE2 is 78 % while BPA only 44 %; thus, EE2 degradation is nearly twice more efficient than BPA.

To obtain reproducibility of the measurements the photocatalytic experiments were repeated and the determined deviation was lower than 5 %. Nevertheless, this deviation includes not only reproducibility of the experiments but also the error of the analytical method and analytical determination. Therefore, it can be concluded that reproducibility of photocatalytic experiments is evidently very good.

Kinetic parameters of EE2 and BPA degradation in photocatalytic reactor were calculated acording the eq. (5) and are summarized in Table II. The obtained kinetic data of EDCs decomposition in the photo-reactor evinced the pseudo first order reaction for both compounds.

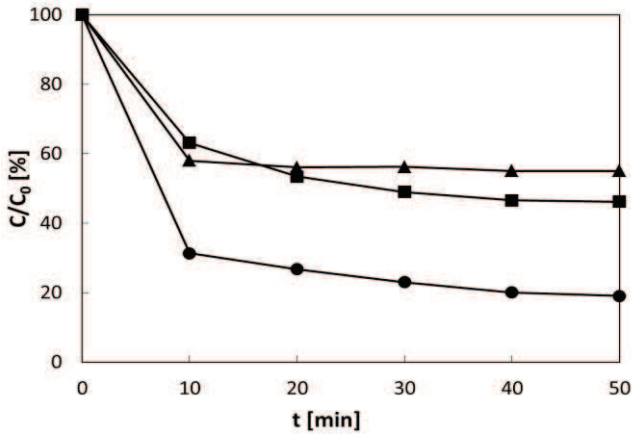

Fig. 4. *Decomposition of BPA from model water at various flow rates, ● – 5 L/h, ■ - 11 L/h, ▲ – 14 L/h*

Comparison of the rate constants (Table II.) clearly shows higher efficiency for EE2 degradation. Degradation of EE2 solution was approximately 3.5 times faster than for BPA solution.

TABLE II. KINETIC PARAMETERS OF EE2 AND BPA SOLUTION DEGRADATED IN PILOT PLANT UNIT

ED	k [min^{-1}]	n [-]	c_{A0} [$mg \cdot L^{-1}$]
EE2	0.091	1.011	11.31
BPA	0.026	1.194	11.83

Many researchers have investigated the degradation of BPA using various series of oxidants and described the oxidation kinetics but only in a laboratory scale [33-35]. In fact, there is a real lack of literature providing similar results for pilot plant scale; nevertheless, J.Sharma [36] studied photolysis of BPA with and without H_2O_2 addition under similar conditions. They achieved without H_2O_2 addition under UV radiation efficiency of 30% during 350 min and with H_2O_2 addition under UV radiation the efficiency increased up to 80% in dependence of H_2O_2 concentration (1.47 -11.76 mM). The reported results for BPA degradation with UV/H_2O_2 showed pseudo-first-order kinetics for the oxidation reactions. Regrettably, important information concerning toxicity and/or estrogenity before and after a degradation process is really missing.

C. The effect of EDCs degradation on toxicity and estrogenity

During photocatalytic degradation of EDCs some new intermediates or by-products are created and these compounds can possess higher estrogenity and toxicity than the original contaminants. Therefore, we focused on estrogenity and toxicity of EE2 and BPA decomposition. The obtained results for the flow rate of 14 L/h are summarized in Fig. 5, where the vertical dashed line represents retention time of the reactor.

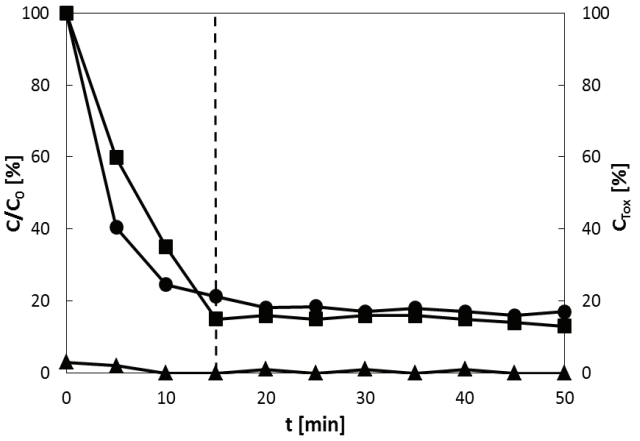

Fig. 5. *Estrogenity and toxicity of EE2 during the photocatalytic experiment, ● – Concentration at 14 L/h, ■ – Estrogenity, ▲ – Toxicity, -- 1 cycle (15 min)*

It is evident that the estrogenic activity of EE2 was reduced to 20 % and the toxicity value ranged from 0 % to 3 %, which falls to error of analytical determination and can be evaluated as a zero toxic effect during the reaction. The measured values of toxicity and the residual concentration in EE2 solution also demonstrated rapid equilibration (15 min) corresponding to the residence time at a given flow. It follows that no emerging products, intermediates or by-products which reveal any estrogenic activity were created.

Similar results were also obtained for BPA degradation. The decrease of estrogenity corresponds to BPA degradation rate and the significant decrease of toxicity was also detected, see Fig. 6. Toxicity and estrogenic activity of BPA solution during the experiment was measured at a flow rate of 5 L/h, which corresponds to the residence time of 45 minutes and the highest degradation efficiency (Fig. 4).

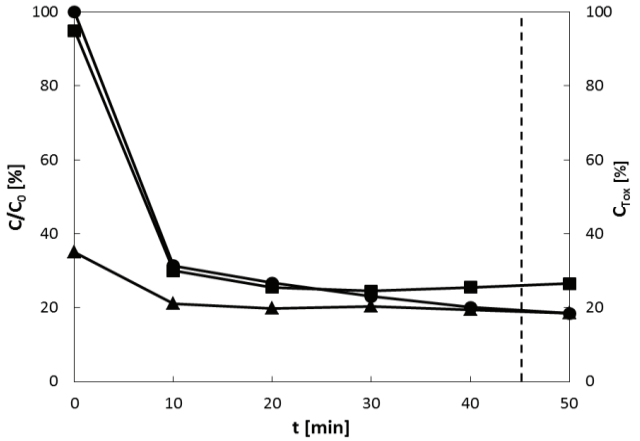

Fig. 6. *Estrogenity and toxicity of BPA during the photocatalytical experiment, ● – 5 L/h, ■ – Estrogenity, ▲ – Toxicity, -- 1 cycle (45 min)*

The results show a significant drop of BPA concentration in the solution during the first 10 minutes, which resulted in concentration and estrogenic activity reduction of 69 % resp. 70% and even the toxicity decreased from 35 % to 20 %. After this period, a slight decrease of BPA residual concentrations to 18.5 % was visible, which could be caused by variation in the flow rate.

These facts corroborate the original idea that during the photocatalytic process in the designed pilot plant unit not only the original compounds but also the intermediates and by-products can be successfully degraded.

D. Degradation of the real waste water

Accumulation of endocrine disruptors in nature has become a significant environmental problem in which municipal and industrial waste water is involved. Therefore, an industrial town with 67 thousand inhabitants was chosen as a source of real waste water. One of the small sewage plants employed for waste water treatment was selected for degradation experiments. The photocatalytic pilot plant reactor was placed as the last purification unit at that sewage plant. Besides the above mentioned contaminants (EE2, BPA, 4-NPH and IRG) were also monitored other common contaminants: estriol (E1), estron (E2) and estradiol (E3). It can be seen (Fig. 7) that concentrations of all contaminants varied in time, particularly concerning BPA and E1. Therefore, long-term photocatalytic experiments were also conducted. Fig. 8 shows the degradation process efficiency for water with a relatively high content of contaminants for the flow rate of 30 L/h with the residence time of 7 min.

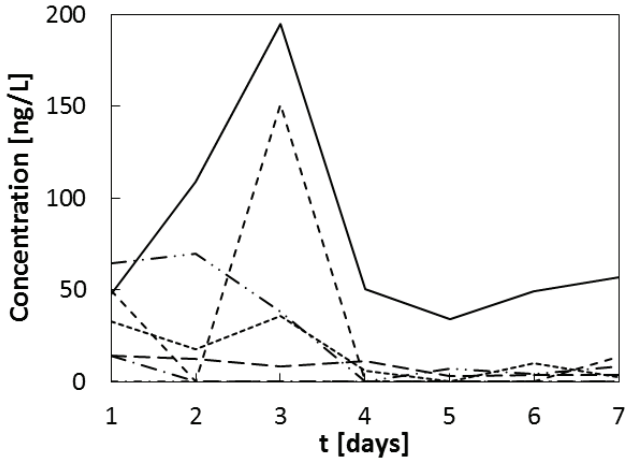

Fig. 7. *Concentration of EDCs during 7 consecutive days, −−− BPA, − − 4-NPH, - - -E1, −·−·E2, − · − E3, −·−·−EE2, -----IRG*

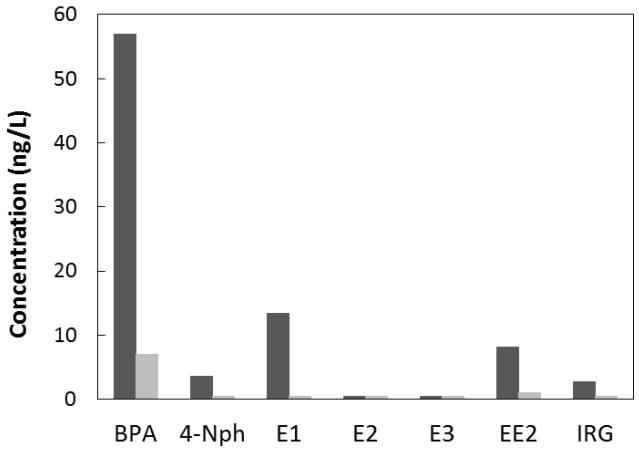

Fig. 8. *Endocrine disruptor degradation from real waste water in the pilot plant reactor (Flow rate 30 L/h; Residence time 7 min), ■ – Input, ■- Output*

Results show that decomposition of 4–Ph, E1 and IRG contaminants achieved more than 95 %, and EE2 and BPA decomposition reached 87 resp. 88 %. E2 and E3 compounds in this waste water sample occurred at minimal concentration only. Thus, a very good degradation efficiency of a pilot reactor was confirmed. High efficiency of the pilot plant reactor should be emphasized, particularly due to the fact that purification of water containing a huge number of mutually influenced contaminants is extremely complicated.

Not only concentration of contaminants but also toxicity and estrogenity determination, and water at the input and output of the photocatalytic pilot plant unit were characterized by the most important indicators of wastewater pollutions, namely TOC (total organic carbon) and COD_{Cr} (chemical oxygen demand). The obtained results are summarized in Table III.

TABLE III. VALUES OF WATER POLLUTION INDICATORS AT THE INPUT AND OUTPUT OF THE PHOTOCATALYTIC REACTOR (5 HOUR)

Water	pH	Conductivity [mS/m]	[1]TOC [mg/L]	[2]TIC [mg/L]	[3]DOC [mg/L]	[4]COD_{Cr} [mg/L]	[5]TSS [mg/L]
Input	7.5	127	9.8	38.9	8.5	62.7	3.8
Output	7.5	129	8.0	41.2	7.5	16.8	3.9

[1]TOC – Total organic carbon, [2]TIC – Total inorganic carbon, [3]DOC – dissolved organic carbon, [4]COD_{Cr} – Chemical oxygen demand, [5]TSS – Total suspended solids

Generally, the process of photocatalytic oxidation (TiO_2/UV) reduced COD_{Cr} and TOC values. It is caused by the non-selective oxidation mechanism in which OH radicals oxidize the entire organic pollution. The value of TOC corresponded to the TOC normal background level in surface waters; therefore, no significant reduction of TOC value was detected. However, there was a significant decrease in COD_{Cr} indicator - almost to the normal background level. De facto, oxidation leads to mineralization of organic substances to targeted inorganic substances, which are CO_2 and H_2O and it causes the increase in TIC content (carbonates and free CO_2). Photocatalytic oxidation may also cause precipitation of certain inorganic materials, which is indicated by the increase in TSS values. In this case, a slight growth of both indicators TIC and TSS was detected, which corroborates the presumptions.

This study also drew attention to the increasing in flow rates and concentration of contaminants. Fig. 9 shows results of degradation process efficiency with a 10 times higher flow rate and 10 times higher concentration of endocrine disruptor pollutants. The same real waste water used for Fig. 8 experiments was spiked by individual contaminants to achieve their concentration between 480 – 550 ng/L. It is evident that even for such high concentrations of contaminants and flow rates 200 L/h or 325 L/h, degradation efficiency of the designed pilot plant reactor reached 93 %, or 88 %, resp.

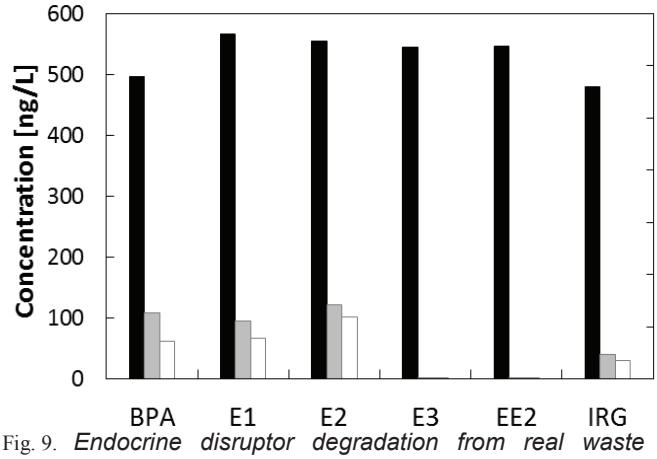

Fig. 9. *Endocrine disruptor degradation from real waste water in the pilot plant reactor (■ input, ■ output 325 L/h, □ output 200 L/h)*

Finely, efficiency of the designed pilot plant reactor was also tested on the rinse water from pharmaceutical industry, which contained another EDCs compounds (Norethisteron, Danazol). Concentrations of both EDCs at the input and output of the photocatalytic reactor for two flow rates (20 L/h and 30 L/h) are summarized in Table IV. It can be seen that degradation efficiency was 90 – 100 % and mainly in all cases it was below the officially allowed limit 0.5 mg/L.

TABLE IV. CONCENTRATION OF NORETHISTERON AND DANAZOL AT THE INPUT AND OUTPUT OF THE PHOTOCATALYTIC REACTOR

	Input	Output	
	-	20 L/h	30 L/h
	c [mg/L]	**c** [mg/L]	**c** [mg/L]
Danazol	5.99	< 0.05	0.1
Norethisteron	4.82	0.106	0.45

IV. CONCLUSIONS

The pilot plant photocatalytic reactor with stainless steel annulets coated by TiO_2 thin layers with the active anatase crystalline structure was designed and successfully tested. For preliminary testing of the designed pilot plant reactor, 17α-ethynylestradiol and bisphenol A as representatives of endocrine disruptors in water solutions of concentration 10 ppm were employed. The photocatalytic degradation of EE2 and BPA was performed at various flow rates in the range of 5 – 14 L/h with efficiency between 78 - 95 % for EE2 and 44 – 80 % for BPA. During the decomposition process, the estrogenity of both simulated waters gradually decreased simultaneously with the degradation of contaminants. The same trend was found for toxicity of BPA solution, while EE2 revealed no toxicity during the whole degradation process. Based on this, it could be deduced that emerging products, intermediates or by-products revealing any estrogenic activity do not arise during the degradation process. These facts corroborate the original presumption that during the photocatalytic process in

this designed pilot plant unit not only the original compounds but also intermediates and by-products can be successfully degraded.

Regarding photocatalytic degradation experiments, the real waste water from an industrial town was chosen to guarantee the presence of various types of EDCs produced from households, hospitals and machinery. Seven main contaminants (EE2, E1, E2, E3, BPA, 4-NPH and IRG) were detected at the output of the sewage plant, which was used as the input into the pilot plant photocatalytic reactor. Long-term photocatalytic experiments were also conducted, due to the fact that concentrations of all contaminants significantly varied in time. The efficiency of the photocatalytic degradation process varied between 87 – 100 % for all photocatalytic experiments. These values of efficiency are significantly high, considering the difficulties connected with purification of water containing a huge number of the mutually influenced contaminants. Moreover, the highest applied flow rate (325 L/h) corresponds to volume of treated water at the smallest sewage plants.

To test the another possibilities of the photocatalytic pilot plant unit utilization, purification of the rinsed water from pharmaceutical industry containing other EDCs compounds (Norethisteron, Danazol) was also performed at two various flow rates; 20 and 30 L/h. Efficiency of the photocatalytic degradation process was 90 – 100 % and it should be emphasized here that the concentration of EDCs dropped down below the officially allowed limit 0.5 mg/L in all experiments.

Finally, it must be mentioned that all experiments were performed at the pilot plant reactor with the original TiO_2 layer, which refers to its enormous resistance and a long life time of the designed photocatalytic unit. It was proved that photocatalytic degradation of endocrine disruptors on TiO_2 layers is an efficient degradation method regarding both the pilot scale and the small real scale.

ACKNOWLEDGMENTS

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Widely used pharmaceuticals present in the environment revealed as *in vitro* antagonists for human estrogen and androgen receptors

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HIGHLIGHTS

- CXCL12-T47D and YES assays showed endocrine disrupting potency of pharmaceuticals.
- Anti-inflammatory drugs exhibited anti-estrogenic and anti-androgenic properties.
- Dose dependency of this endocrine disrupting effect has been proved.
- The results can explain some mechanisms of adverse effects on humans and wildlife.

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Recombinant yeast assay

ABSTRACT

A considerable amount of scientific evidence indicates that a number of pharmaceuticals that could be detected in the environment can contribute towards the development of problems associated with human reproductive systems, as well as those of wildlife. We investigated the estrogenic and androgenic effects of select pharmaceuticals with high production volume and environmental relevance. We examined the receptor-binding activities of these pharmaceuticals in the T47D human cell line using altered secretion of cytokine CXCL12. Functional yeast-luciferase reporter gene assays were also employed to confirm the mechanism of receptor binding by estrogen and androgen. Non-steroidal anti-inflammatory drugs, namely ibuprofen, diclofenac and antiarrhythmic agent amiodarone showed strong anti-estrogenic effects in the T47D cell line.

In the yeast-luciferase assay, these anti-inflammatory drugs also demonstrated anti-estrogenic potency and inhibited the E2 response in a concentration-dependent manner. Amiodarone did not exhibit any response in the yeast-luciferase assay; therefore, the endocrine disruption presumably occurred at a different level without directly involving the receptor. All the anti-inflammatory drugs considered in this study, including ketoprofen, naproxen and clofibrate, exhibited a dose-dependent antagonism towards the androgen receptor in the yeast-luciferase assays. Several other drugs, including the stimulant caffeine, did not show any response in the tests that were employed.

A risk assessment analysis using 'Hazard Quotient' suggested a potential risk, especially in the cases of ibuprofen, ketoprofen, diclofenac and clofibrate.

The results reveal the intrinsic endocrine disrupting nature of several pharmaceuticals and thus could contribute towards explaining a number of adverse health effects on humans and wildlife.

1. Introduction

Pharmaceuticals and personal care products (PPCPs) are an emerging class of anthropogenic environmental contaminants that are drawing increasing attention. Large quantities of pharmaceuticals used in human and veterinary medicine, such as beta-blockers, anti-inflammatory drugs, contraceptives, antibiotics, lipid regulators, neuroactive compounds and many others, are sold and consumed worldwide either for treatment or for diagnosing diseases (Khetan and Collins, 2007). Consequently, an increasing number of studies are also reporting the detection of pharmaceutical compounds in both wastewater and surface waters in the

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range of nanograms to milligrams per liter (Daughton and Ternes, 1999; Fick et al., 2009; Rodil et al., 2012; Tixier et al., 2003; Vystavna et al., 2012); it is also detected in the wild fish population (Brozinski et al., 2013). Some articles evaluated the risk associated with PPCPs or other chemicals, such as plasticizers (Barry et al., 2005; Tharpa et al., 2012).

Pharmaceuticals are designed to be bioactive; thus, many of them acts as baseline toxicants in non-target aquatic life. However, some of them also exhibit a more severe biological effect on aquatic life, such as that which was reported in fish regarding the undesirable effects of estrogen (Kidd et al., 2007; Rostkowski et al., 2011; Escher et al., 2011). On a similar note, for instance, Stancová et al. (2015) showed that naproxen, at an environmentally relevant concentration of 1 μg/mL, significantly influenced the mRNA expression level of three genes related to oxidative stress in the intestine of zebrafish.

Although several studies have suggested that commonly used pharmaceuticals could interact with the sexual functions of human and animals (see e.g., Khetan and Collins, 2007 and references therein), very few studies have dealt with the possible endocrine activity (estrogenic or androgenic) of either the pure compounds (drugs) or the environmental samples related to modern pharmaceutical industry. This question is not only important for environmental safety, but it is also relevant to human toxicology and the adverse side effects of drugs.

Due to the large amount of pharmaceuticals used as personal care products and in hospitals, there is an urgent need for listing and ranking of compounds of high concern (Escher et al., 2011; Jean et al., 2012). This is not an easy task because the environmental concentrations of pharmaceuticals may vary in different regions with contrasting socio-economic conditions (Vystavna et al., 2012).

Therefore, better data are needed to determine which pharmaceuticals could potentially pose the highest risk to consumers and to the environment.

For our study, we chose pharmaceuticals with the high volumes of production that have already been detected in the aquatic environment and in wild animals (Brozinski et al., 2013; Vystavna et al., 2012; Leung et al., 2013). We selected four non-steroidal anti-inflammatory drugs, namely ibuprofen, diclofenac, ketoprofen and naproxen as well as the antipyretic acetaminophen, the anticonvulsant carbamazepine, the anti-arrhythmic agent amiodarone, the fibrate clofibrate, the beta blocker atenolol and the central nervous system stimulant caffeine. An increasing amount of evidence indicates that some of these pharmaceuticals might possess endocrine-disrupting potency. Some non-steroidal anti-inflammatory agents, including naproxen and ibuprofen, were reported to inhibit estrogen sulfotransferase (King et al., 2006), whereas other authors have suggested that ibuprofen might alter steroidogenesis (Han et al., 2010). Diclofenac has been demonstrated to cause erectile dysfunctions in experimental male rats (Senbel, 2011). Clofibrate was also demonstrated to have some potency for disruption, acting via decreases in the estrogen and androgen receptor mRNA level, in an experiment using F344 rats (Fujimoto et al., 2012). Suppression of spermatogenesis was observed in both dogs and monkeys treated with clofibrate (Cook et al., 1999). Some articles reported that amiodarone might cause gynecomastia as an adverse effect (Krause, 2012). All these deleterious effects could be mediated by the estrogen or androgen receptor pathway.

In vitro assays with human cell lines or recombinant yeasts are widely used tools to measure and demonstrate the estrogenic activity of various compounds (Matsushima et al., 2010; Orton et al., 2011; Wang et al., 2012). In this study, we determined the agonistic and antagonistic interactions between commonly used pharmaceuticals and human estrogen and androgen receptors, using two *in vitro* bioassays – T47D cell line linked with ELISA CXCL12 (also called SDF1, referring to Stromal cell Derived Factor 1) assay and a recombinant yeast assay with strains BMAERElu c/ERα and BMAERElu c/AR – to comprehensively evaluate the estrogenic potencies of test compounds. We focused on the anti-estrogenic properties of the selected compounds and evaluated them in relation to known anti-estrogens. The observed endocrine disruptive activities, which are related to the concentrations of the test compounds, were used further for a risk assessment analysis and were estimated using hazards coefficients (HQ).

2. Material and methods

2.1. Chemicals

Ibuprofen (≥98%, GC), naproxen (≥98%, USP Reference Standard), ketoprofen (≥98%), acetaminophen (≥99%), carbamazepine (USP Reference Standard), amiodarone hydrochloride (≥98%), clofibrate (≥98%), atenolol (≥98%), caffeine (≥99%), fulvestrant (>98%, HPLC), 2,4,6-tribromophenol (TBP, 99%), testosterone (Tes, ≥99%) and 17β-estradiol (E2, 98%) were obtained from Sigma–Aldrich (Germany). All the tested chemicals were dissolved and diluted in a mixture of dimethyl sulfoxide (DMSO; ≥99.9%, Sigma–Aldrich Germany) and Milli-Q water (30/70, v/v). For the CXCL-test, the compounds were dissolved and diluted in ethanol (Penta, Czech Republic). Synthetic D-luciferin was obtained from Biotech a.s. (Czech Republic).

2.2. Cell culture and CXCL-test

The human T47D breast carcinoma cell line was kindly provided by Dr. Truksa, Laboratory of Tumor Resistance, Academy of Sciences of the Czech Republic. T47D cells were routinely maintained in RPMI (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and antibiotics (Invitrogen) at 37 °C in 5% CO2. The assay was performed according to Habauzit et al. (2010). Briefly, the cells were maintained in phenol red-free RPMI (Invitrogen) and supplemented with 5% charcoal-stripped fetal bovine serum when treatment with steroids were required. For all the experiments, T47D cells were plated in 24-well plates with 7×10^4 cells/well in phenol red-free RPMI with 5% charcoal-stripped FBS.

A total of 1 μl of solutions of the test compounds at various concentrations (Table 1) were added to the wells in such a way that the concentration of ethanol in the cell cultures did not exceed 0.1%. All the tests were performed in triplicate. The following controls were carried out on each plate: media, ethanol, E2 (9.18 pM).

In response to ER agonists, the cells secreted CXCL12/SDF1, and the full logistic curve for E2 was obtained in which 9.18 pM of E2 reached 80% of the maximal response. The cells were incubated for 2 days at 37 °C in 5% CO_2, then culture supernatants of each triplicate were pooled together and immediately measured by the enzyme-linked immunosorbent assay (ELISA). The ELISA test was performed with a Quantikine kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The absorbance at 450 and 570 nm was measured using a 96-well microplate reader (Tecan Infinite Pro, USA).

For the anti-estrogenic assay, 9.18 pM (EC_{80}) of E2 were added together with the tested compounds, and the modification or changes in the secretion of CXCL12 was monitored (Teng et al., 2013). For comparative purposes, the data were normalized to E2 alone at the exposure concentration (response of 80% of the maximum) and for solvent-only (ethanol) controls (minimum response, 0%). Fulvestrant and 2,4,6-tribromophenol were used as controls because of their known anti-estrogenic effects. The anti-estrogenic potency of 2,4,6-tribromophenol was demonstrated

Table 1
The detected anti-estrogen and anti-androgen activities of the studied pharmaceutical compounds and the determined or calculated IC_{50} values.

Trivial name	Systematic name (IUPAC)	Type of drug	T47D human cell line tested concentration range or highest tested concentration (μM)	Yeast assays tested concentration range or highest tested concentration (μM)	T47D human cell line IC_{50} of anti-estrogenic effect (μM)	Yeast assays IC_{50} of anti-estrogenic effect (μM)	Yeast assays IC_{50} of anti-androgenic effect (μM)
Ibuprofen	(*RS*)-2-(4-(2-methylpropyl) phenyl)propanoic acid	anti-inflammatory	15–484	0.20–96.14	311.3	22.2	29
Diclofenac	2-(2-(2,6-dichlorophenylamino) phenyl)acetic acid	anti-inflammatory	10–337	0.28–70.35	63.09	9.45	17.6
Ketoprofen	(*RS*)-2-(3-benzoylphenyl) propanoic acid	anti-inflammatory	12–393	0.33–65.54	ND	100.00[a]	6.76
Naproxen	(+)-(*S*)-2-(6-methoxynaphthalen-2-yl) propanoic acid	anti-inflammatory	6–217	0.18–72.38	ND	80.02[a]	92.73[a]
Amiodarone	(2-{4-[(2-butyl-1-benzofuran-3-yl)carbonyl]-2,6-diiodophenoxy}ethyl) diethylamine	anti-arrhythmic	0,15 - 31	1.41	10.59	ND[b]	ND
Clofibrate	ethyl 2-(4-chlorophenoxy)-2-methylpropanoate	fibrate	12–412	0.34–148.33	ND	170.84[a]	11.62
Acetaminophen	*N*-(4-hydroxyphenyl) ethanamide	antipyretic	661	120.27	ND	ND	ND
Carbamazepine	5*H*-dibenzo[*b*,*f*]azepine-5-carboxamide	anticonvulsant	105	76.95	ND	ND	ND
Atenolol	(*RS*)-2-{4-[2-Hydroxy-3-(propan-2-ylamino) propoxy]phenyl}acetamide	beta blocker	187	20.48	ND	ND	ND
Caffeine	1,3,7-trimethyl-1*H*-purine-2,6(3*H*,7*H*)-dione	central nervous system stimulant	8	93.63	ND	ND	ND

[a] Due to insufficient solubility, the IC50 values for these compounds were calculated via sigmoid fitting using OriginPro software.
[b] Not detected.

previously (Ezechiáš et al., 2012). To evaluate possible toxic effects on the cell line, the cultures on each 24-well plate were trypsinized, re-dissolved in the medium, immediately mixed with trypan blue stain (0.4%, Invitrogen, USA) and measured with an automatic cell counter (Invitrogen, USA).

2.3. Bioluminescent estrogen and androgen screens

The estrogen and androgen-like activities of the tested chemicals were also measured using a recombinant strain of *Saccharomyces cerevisiae*. The bioluminescent yeast strains *S. cerevisiae* BMAEREluc/ERα, *S. cerevisiae* BMAEREluc/AR, and *S. cerevisiae* BMA64 luc described by Leskinen et al. (2005) were used. The tests were carried out according to Leskinen et al. (2005) as described previously (Ezechiáš et al., 2012). Briefly, stimulation of the transfected estrogen and androgen receptor causes secretion of luciferase into the culture medium. The growing yeast strain was incubated with the test compounds at 30 °C for 2.5 h. The culture was then shaken briefly and D-luciferin was added to the medium and immediately measured using a Lumino-M90a luminometer (ZD Dolní Újezd, Czech Republic) with 60 s integration time for the luminescence detection. All measurements were performed in triplicate. The BMA64 luc strain served as a control for the cytotoxicity of the tested compounds in these tests. The tests were performed in stock solutions that were diluted 11 times with media containing the respective yeast strain so that the concentration of DMSO in the medium did not extend 3%. Serial dilutions of E2 and Tes for BMAEREluc/ERα and BMAEREluc/AR, respectively, were run as a positive control in each experiment. The anti-hormonal activities were evaluated by testing the ability of the chemicals to diminish the yeast response evoked by E2 and Tes in the presence of the tested compounds, expressed as a percentage of the test response to the positive controls containing E2 (4.16×10^{-4} mg/L) and Tes (4.16×10^{-3} mg/L) for the anti-estrogen and anti-androgen assays, respectively (Teng et al., 2013). A total of 3% DMSO served as a blank for all the tests, and the cultures were also cultivated without the addition of DMSO to evaluate its possible toxic effect.

2.4. Data analysis

All the results are expressed as the mean ± SD. The difference was considered significant at p value ≤ 0.05. The concentration–response analyses were performed with four-parameter logistic curve fitting analysis according to the following formula:

$$y = y_{max} + (y_{min} - y_{max}) \Big/ \left[1 + (x/x_0)^h\right]$$

where y is the response value, x is the concentration of the test compound, x_0 is the inflection point of the curve usually representing the EC_{50} concentration that induces half of the maximum effect, and h is a parameter that corresponds to the slope of the curve. The data from ELISA readings were normalized on a plate-by-plate basis to the means of the solvent controls and positive E2 controls (n = 6). The corrected values of the absorbance and luminescence for the CXCL-test and yeast assay, respectively, versus the concentrations of the chemicals expressed in common logarithmic scale were plotted for each chemical. The nominal IC_{50} values were calculated from two independent experiments (both performed in triplicate) as the nominal chemical concentrations at which 50% of the test response inhibition is reached. All the statistical analyses were performed and the determination of IC_{50} was processed with OriginPro 8.5 (OriginLab, USA) software.

HQ was calculated as the ratio between predicted non-effect concentrations (PNEC) and environmental concentrations of the pharmaceuticals with detectable endocrine activities (Mendoza

et al., 2015; Ginebreda et al., 2010). PNEC were estimated using determined IC_{50} values from the dose–response curves after correction by an assessment factor of 1000 (European Commission, 2003).

3. Results

3.1. CXCL12 test

T47D cell line linked with the ELISA assay was used to investigate the estrogenic activities of the selected pharmaceuticals. As expected, E2 induced a significant increase in the CXCL12 secretion, but none of the pharmaceuticals triggered CXCL12 secretion above 10% of the maximal E2 response, even at the highest tested concentrations. Therefore, we could not construct any sigmoidal response curves for the agonists. On the other hand, ibuprofen, diclofenac and amiodarone showed strong inhibitory effects in the anti-estrogenic experiment. Therefore, a range of concentrations of these compounds that were compatible with the assays were measured to demonstrate the dose-dependence nature of the effects. The selective estrogen receptor inhibitor fulvestrant and the environmental estrogen antagonist 2,4,6-tribromophenol were also measured and used as positive controls. The obtained sigmoidal curves are plotted in Fig. 1, and the nominal IC_{50} values of single compounds are listed in Table 1. Fulvestrant, as the selective estrogen receptor antagonist, had the highest potency to inhibit the E2 response. The nominal IC_{50} for fulvestrant was 1.63×10^{-4} μM. Among other test compounds, the most potent *in vitro* antagonist was amiodarone, reaching IC_{50} at 10.59 μM. Amiodarone was followed by 2,4,6-tribormophenol, diclofenac and ibuprofen with their IC_{50} values of 39.6 μM, 63.09 μM and 311.3 μM, respectively. The other tested pharmaceuticals did not demonstrate any significant dose-dependent inhibitory effect.

To exclude any cytotoxic effect of the tested pharmaceuticals, the cells were trypsinized after removing the medium and their viability was quantified using an automated cell counter. In all cases the viability did not decrease below 80%, even with the highest tested concentrations.

3.2. Recombinant yeast assay

None of the tested compounds under study triggered any agonistic response of the estrogen receptor in the yeast assay. The only exception was a minor estrogenic response associated with ketoprofen and clofibrate when applied at their respective highest concentrations, (t-test, $p < 0.05$). Furthermore, the estrogenic effects of these compounds were so low that dose-dependent sigmoid curves could not be constructed. In the case of androgen receptors, a nominally significant agonistic effect was recorded with the highest concentration of clofibrate tested in our study (t-test, $p < 0.05$).

On the other hand, all the tested anti-inflammatory agents and clofibrate exhibited substantial anti-estrogenic and anti-androgenic activity. Therefore, also in this case, a wide range of concentrations of these compounds (see Table 1) compatible with the assays were measured to demonstrate the dose-dependence. The obtained sigmoidal curves which documents the inhibition are plotted in Figs. 2 and 3, while the nominal IC_{50} values of single compounds are listed in Table 1. The highest anti-estrogenic potency was observed in the presence of diclofenac ($IC_{50} = 9.45$ μM), while 22.2 μM of ibuprofen were required to inhibit the estrogen receptor by one half. We were able to create a sigmoidal dose–response curve in a satisfactory concentration range to reach the inflection point representing IC_{50} for both of these chemicals. Ketoprofen, naproxen and clofibrate also acted as inhibitors of the estrogen receptor in a concentration-dependent manner. However, the IC_{50} values of these three compounds, which are 100.00, 80.02 and 170.84 μM, respectively, were calculated *via* sigmoid fitting function of the OriginPro software because we could not determine these values directly from our experimental conditions. Fulvestrant as a selective estrogen receptor antagonist reached a nominal IC_{50} at 3.3 μM, which is much higher than the IC_{50} value obtained from the CXCL12 test.

The same compounds also exhibited anti-androgenic effects, and their IC_{50} values are displayed in Table 1. Ketoprofen had the greatest ability to inhibit the androgen receptor response among all the tested compounds, reaching IC_{50} at 6.76 μM. In contrast, naproxen was the least potent anti-androgenic compound among the other anti-inflammatory drugs, reaching maximal inhibition of the testosterone (Tes) test response of 42.8% at a concentration of 72.38 μM. Higher concentrations (also in the other cases) could not be measured due to the insufficient solubility of the compound in the 30% DMSO stock solution used in the test. Therefore, the IC_{50} value for naproxen was also calculated *via* sigmoidal fitting using the OriginPro software, yielding a value of 92.73 μM naproxen. The

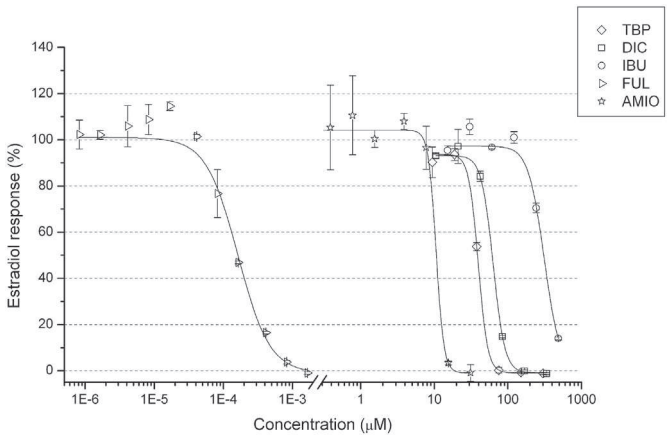

Fig. 1. The anti-estrogen activity of 2,4,6-tribromophenol (TBP), diclofenac (DIC), ibuprofen (IBU), amiodarone (AMIO) and fulvestrant (FUL) determined by the CXCL12-test. The estradiol response corresponds to the induction of the sensors caused by 9.18 pM of 17β-estradiol. 100% represents no inhibition of the 17β-estradiol response. The Y error bars represent the standard deviations.

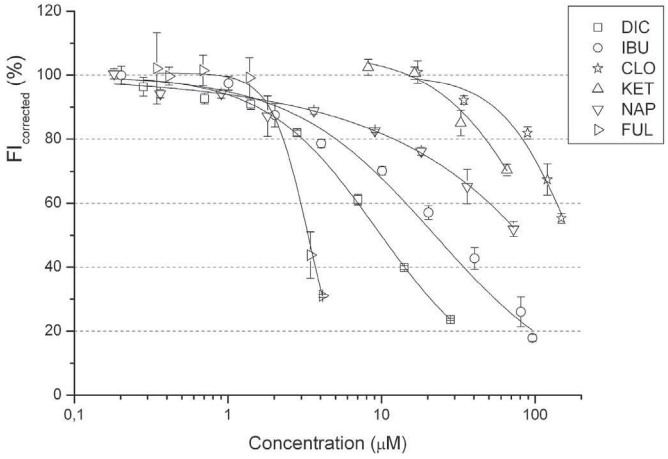

Fig. 2. The anti-estrogen activity of diclofenac (DIC), ibuprofen (IBU), ketoprofen (KET), naproxen (NAP), clofibrate (CLO) and fulvestrant (FUL) determined by the bioluminescent assay. $FI_{corrected}$ represents the induction of the sensors corrected for the sample toxicity. 100% represents no inhibition of the 17β-estradiol response. The Y error bars represent the standard deviations.

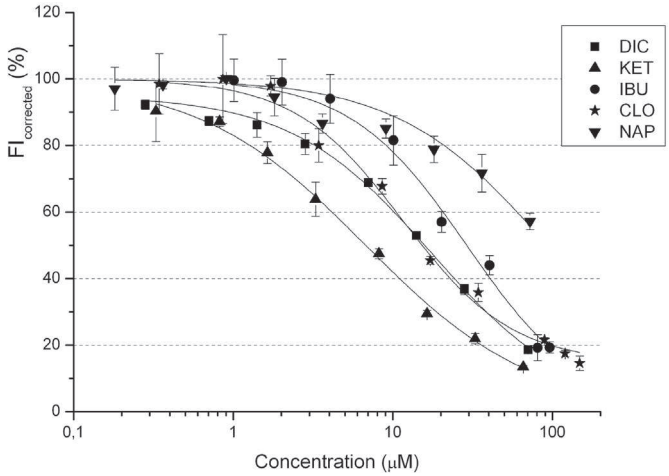

Fig. 3. The anti-androgen activity of diclofenac (DIC), ibuprofen (IBU), ketoprofen (KET), naproxen (NAP) and clofibrate (CLO) determined by the bioluminescent assay. $FI_{corrected}$ represents the induction of the sensors corrected for the sample toxicity. 100% represents no inhibition of the testosterone response. The Y error bars represent the standard deviations.

other tested compounds, such as carbamazepine, atenolol, acetaminophen, caffeine and amiodarone (Table 1), did not show any significant agonistic or antagonistic effects on the receptor (t-test, $p < 0.05$) in the yeast reporter genes assay.

3.3. Environmental risk

After the quantification of the hitherto unreported adverse effects of some of the drugs under the present study, HQs were calculated using PNEC values and environmental concentrations from various aquatic environments published in several recent reviews and a study dealing with hospital wastewater: see Lapworth et al. (2012); Mendoza et al. (2015); Murray et al. (2010); and Verlicchi et al. (2012). The environmental concentrations, the results of PNEC and HQ of anti-estrogenic and anti-androgenic effects are shown in Table 2.

4. Discussion

Our results indicate that a systematic testing of anti-estrogenic and anti-androgenic activity of various compounds is desirable even for the currently used pharmaceuticals. For example, two anti-inflammatory drugs, ibuprofen and diclofenac, which have high consumption volumes worldwide, were found to have a substantial inhibitory effect towards estrogen and androgen receptors. The endocrine-influencing potency of ibuprofen had been studied previously using H295R human adrenocarcinoma cells (Han et al., 2010). It was reported that ibuprofen significantly increased the production of E2 and decreased testosterone concentration in H295R cells in a dose-dependent manner. The authors also suggested that ibuprofen could have altered the steroidogenesis and, supported by the results from our present study, it appears that normal hormonal signaling can be disrupted by ibuprofen at several levels. Fernandes et al. found that pharmaceutical compounds, such as ibuprofen, diclofenac and clofibrate, inhibit C17, 20-lyase and CYP11b, i.e., enzymatic activities involved in the synthesis of active androgens (Fernandes et al., 2011). Several non-steroidal anti-inflammatory agents including naproxen and ibuprofen were also demonstrated to inhibit estrogen sulfotransferase (SULT1E1), which is involved in the attenuation of steroid hormone signaling (King et al., 2006).

Some studies reported that the use of nonsteroidal anti-inflammatory drugs also increases the incidence of erectile dysfunction in the human population (Gleason et al., 2011; Shiri et al., 2006). This negative effect has been demonstrated for diclofenac in experimental measurements of erectile responses in male rats (Senbel, 2011).

These evidence of adverse effects might be explained by the interaction of the pharmaceutical compounds with the steroid receptor. Our results demonstrated that these anti-inflammatory drugs act as inhibitors of the estrogen receptor pathway and are antagonistic towards estrogen and androgen receptors. The most potent antagonist for the estrogen receptor is diclofenac in both the tests described in this article, followed by ibuprofen.

Amiodarone demonstrated strong inhibition of estrogen receptor signaling in the CXCL12-test using the human cell line, but it did not exhibit any interaction with the estrogen receptor pathway in the yeast reporter gene assay. Signaling by steroid hormone is mediated by receptor proteins that bind hormonal ligands and regulate gene transcription. It is known that heat-shock protein hsp82 interacts selectively with the steroid receptor and can alter the receptor function (Picard et al., 1990). Hormonal activation of the estrogen receptor is strictly dependent on the level of hsp82 expression. Various hormonal ligands that activate receptor-mediated transcriptional enhancement produced little or no effect when hsp82 expression was repressed (Picard et al., 1990). It is also known that proteins of the hsp90 family share only 50% amino-acid identity for the most distantly related eukaryotes (Lindquist and Craig, 1988). Other factors, such as p23, also play an important role for estrogen receptor transcriptional enhancement (Knoblauch and Garabedian, 1999). Amiodarone may alter the expression of genes or otherwise interact with these factors and cause disruption of the estrogen receptor transcriptional pathway. Further studies should be performed to investigate the specific mechanism of amiodarone mediated disruption of estrogen signaling. Amiodarone has also been studied for its ability to disrupt the thyroid hormone transcriptional pathway. Li et al. (2008) demonstrated that amiodarone hydrochloride significantly inhibited the triiodothyronine activity at a concentration of 1×10^{-8} M or greater in experiments using the yeast assay (Li et al., 2008). However, another study using a transfected human cell line to measure the thyroid receptor activity demonstrated that amiodarone did not antagonize the response induced by triiodothyronine (Freitas et al., 2011). This contradiction only supports the hypothesis that amiodarone interacts with factors involved in hormone signaling other than direct inhibition of the hormone receptor.

Inhibition of the transcriptional activity of the estrogen and androgen receptor genes following exposure to clofibrate has already been studied. Fujimoto et al. demonstrated that the levels of the estrogen and androgen receptor mRNAs were reduced after the addition of clofibrate in an experiment using F344 rats (Fujimoto et al., 2012). Clofibrate was also tested with a similar reporter gene yeast assay, but the compounds did not show any significant estrogenic response (Ashby et al., 1997); however, the authors only monitored the agonistic effect of the drug. On the other hand, clofibrate also has a number of other negative side effects on reproduction that could probably be linked with androgen receptor inhibition. Some of the effects include impotence, decreased libido and arrest of spermatogenesis (RxList, 2012). Suppression of spermatogenesis was observed in both dogs and monkeys treated with clofibrate at dosage ranging between 160 and 240 mg/kg; moreover, clofibrate was found to induce Leydig cell tumors in rats at a dosage of 400 mg/kg (Cook et al., 1999). It has been reported that clofibrate interacts *via* the inhibition of enzymes involved in the synthesis of active androgens

Table 2
The hazard quotients (HQ) calculated according to European Commission (2003) using concentrations in various aquatic environments and IC_{50} values determined with the yeast assays (anti-estrogenic and anti-androgenic effects).

Drug compound	Concentration (μg/L) [maximum concentration]	PNEC (μg/L) anti-estrogenic effect	HQ anti-estrogenic effect	PNEC (μg/L) anti-androgenic effect	HQ anti-androgenic effect
Freshwater environment (Murray et al., 2010)					
Ibuprofen	0.006 [31]	4.58	0.00	5.98	0.00
Diclofenac	0.15 [1.2]	2.80	0.05	5.21	0.03
Ketoprofen	0.01 [0.24]	25.43	0.00	1.72	0.01
Naproxen	0.004 [2.0]	18.43	0.00	21.35	0.00
Clofibrate	0.066 [0.55]	41.46	0.00	2.82	0.02
Groundwater environment (Lapworth et al., 2012)					
Ibuprofen	1.491 [12]	4.58	0.37	5.98	0.25
Diclofenac	0.121 [0.59]	2.80	0.04	5.21	0.02
Ketoprofen	0.611 [2.886]	25.43	0.02	1.72	0.36
Clofibrate	1.113 [7.3]	41.46	0.03	2.82	0.40
Effluent of the WWTP (Verlicchi et al., 2012)					
Ibuprofen	3.7 [48.2]	4.58	0.79	5.98	0.60
Diclofenac	0.8 [10.7]	2.80	0.29	5.21	0.15
Ketoprofen	0.36 [2.27]	25.43	0.01	1.72	0.21
Naproxen	1 [5.2]	18.43	0.05	21.35	0.05
Clofibrate	0.3 [0.8]	41.46	0.01	2.82	0.11
Hospital wastewater (Mendoza et al., 2015)					
Ibuprofen	1.425 [2.196]	4.58	0.31	5.98	0.24
Diclofenac	0.614 [0.676]	2.80	0.22	5.21	0.12
Ketoprofen	2.46 [4.233]	25.43	0.10	1.72	1.43
Naproxen	2.224 [7.095]	18.43	0.12	21.35	0.10

in male fish (Fernandes et al., 2011). Another article reported toxic properties of a clofibrate metabolite, i.e., clofibric acid, in aquatic environments (Runnalls et al., 2007), where it was demonstrated that the sperm count was significantly decreased by increased concentrations of clofibric acid in fishes. Spermatogenesis is especially an androgen-dependent process; hence, one explanation for the impaired spermatogenesis is exposure to clofibric acid or clofibrate, which reduces androgen synthesis and inhibits the androgen receptor.

The bioavailability and pharmacokinetics of these drugs has already been discussed in various articles. Mustofa et al. (1991) reported a mean concentration of 2791 ng/mL in the blood of healthy volunteers following oral administration of 50 mg of diclofenac. This value corresponds to 9.42 μM after recalculation and is very close to the IC_{50} of the anti-estrogenic effect of diclofenac, as measured in our study (9.45 μM in yeast assay and 63.09 μM in CXCL12-test). Dewland et al. (2009) studied the bioavailability of ibuprofen after a single dose of 2 × 200 mg. The authors recorded a maximum concentration of 32.84 μg/mL in plasma (arithmetic mean from 22 volunteers), which corresponds to 159.19 μM. This value is substantially higher than that of our measured IC_{50} in the yeast assay. Other authors focused on the bioavailability of ketoprofen in various species. The maximum plasma concentration for pigs reached 7.26 μg/mL, which corresponds to 28.55 μM after recalculation. This concentration is more than 4 times higher than our observed IC_{50} for the anti-androgenic effect of ketoprofen (Neirinckx et al., 2011). In his study, Chasseaud et al. detected a peak of the clofibrate concentration in plasma reaching 55 μg/mL (Chasseau et al., 1974). This value (226.62 μM after recalculation) is almost 20 times higher than the IC_{50} value obtained for the anti-androgenic activity in our study.

According to our results, as displayed in Table 2, the environmental risks related to the endocrine-disruptivity of the drugs is limited. Calculated HQs were close to 1 or higher only in few cases; when HQ values are equal to 1 or higher, it is associated with a situation of elevated environmental risk. If HQ is found in the interval of 0.1 and 1, the risk is usually classified as low but potential (European Commission, 2003). Generally, the calculated HQ values in fresh waters within this study were below 0.1. Most of the data reviewed by Murray et al. (2010) that were used for our calculation originated from the USA and the concentration levels were in good agreement with more recent data published e.g., by Arlos et al. (2015). The highest HQ value was found in the case of ketoprofen and its anti-androgenic effect in hospital wastewater (Valencia Region in Spain; HQ = 1.4; Mendoza et al., 2015). Regarding this compound and its anti-androgenic effect, we detected a potential risk (HQ = 0.2) in the case of average of the WWTP effluents from 21 samples around the world (Verlicchi et al., 2012). An even higher value (0.4) was calculated using an average value of groundwater samples from Western Europe and North America (Lapworth et al., 2012). Elevated HQ levels were also found for ibuprofen that reached anti-androgenic and anti-estrogenic values 0.6 and 0.7 in the WWTP effluents, respectively. The WWTP effluent average concentration includes data summarized by Verlicchi et al. (2012), originating mainly from Europe, the USA, Japan and Australia. Regarding the environmental data, it is noteworthy that the calculations were performed with the mean values of the drug concentrations. However, taking into account the displayed maximum concentrations, the estimated HQ values can increase dramatically.

5. Conclusion

To our knowledge, this is the first study documenting the potential of anti-inflammatory pharmaceuticals to directly inhibit human androgen and estrogen receptors in a concentration-dependent manner. Due to their endocrine-disrupting potential, demonstrated here by the use of the reporter-gene assay and CXCL12-test based on a human cell line, these pharmaceuticals could alter the synthesis of active hormones and inhibit hormone-dependent responses in human cells. These pharmaceuticals are typically present in the environment and are readily administered in mixtures; therefore, human exposure to mixtures of these *in vitro* anti-estrogens may be considerable. The IC_{50} concentrations found in this article are in at a level of mg/L that are substantially higher than the typically detected mean concentrations in aquatic environments. However, the risk assessment using HQ indicates a potential risk that could be assigned to several of the drugs. The findings of this paper emphasize the need for further research

regarding hormonal disrupting properties of these pharmaceuticals and also the need to monitor the concentrations of these chemicals in the environment due to their newly discovered adverse effects. These results also pose a question about the possible effects of these drugs on hormone-dependent tumors expressing estrogen or androgen receptors. Moreover, the results also document that these *in vitro* tests could be a useful tool for examining the direct effect of drugs or other compounds on specific receptors.

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Novel full logistic model for estimation of the estrogenic activity of chemical mixtures

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ABSTRACT

Estrogenic compounds as well as other biologically active substances are commonly present in the form of complex mixtures in the environment. There is still no satisfactory model that would be capable of predicting the toxic effects of mixtures containing partial receptor agonists and compounds with different parameters of their dose-response curves. Therefore, a novel Full Logistic Model (FLM) of prediction using all the parameters of dose-response curves has been suggested and compared with previously published approaches. We tested the receptor-binding activities of selected estrogens including full and partial agonists and their mixtures using yeast reporter gene assays and the human T47D cell line. Combination effects were modeled with FLM and predicted curves were compared with the data obtained experimentally. FLM yielded a good fit to the experimental data from both the receptor-binding assays and gave better predictions than the previously published approaches. FLM also provided satisfactory results regarding final partial agonistic dose-response curves with maximum influenced by the inhibitory effect of the partial agonist. FLM is not limited by any simplification like the toxic equivalency factor approach or generalized concentration addition and therefore it could be employed for mixtures containing chemicals with different parameters of their dose-response curves (maximum, minimum, inflex point or slope).

1. Introduction

Estrogenic compounds are widely detected in the environmental samples including waters from treatment plants, wastewaters sludge, surface waters, etc. These compounds in combination with other micropollutants can result in complex mixtures that can exhibit a greater effect than individual compound alone. More than 95% of toxicological research studies are focused on single chemicals and almost completely neglect mixtures (Kortenkamp, 2007). Prediction of mixture effects is a great challenge, because synergism or antagonism in a combination of two or more drugs may occur and no currently available mathematical model can predict and solve this problem fully.

Concentration addition (CA or synonymously dose addition) is a widely used toxicological and pharmacological concept for prediction of the mixture effects of chemicals with a similar mode of action when only the toxicities of the individual components are known (Scholze et al., 2014). The concept of CA (Eq. (1)) estimates mixture effects according to the original mathematical formulation described by Loewe (Loewe and Muischnek, 1926).

$$\sum_{i=1}^{n} \frac{d_i}{ED_i} = 1 \quad (1)$$

where d_i is the dose of compound i in the mixture that produces a toxic effect and ED_i is the dose of the individual components that on their own produce the same effect as the mixture. The sum in this equation is always considered to be equal to 1, i.e. the individual compounds in the mixture simply act additively with no synergistic or antagonistic (supra- or infra-additive) effects. The quotients d_i/ED_i are called toxic units for the individual compounds (Scholze et al., 2014).

At the present time, several methods are available for evaluating combined exposures that are derived from the concept of CA. The toxic equivalency factor (TEF) approach described by Safe (1998a,b) is the most studied and published method. This approach assumes that one compound in the mixture can be replaced by a proportional amount of another compound in terms

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of their potency. This could be described by Eq. (2).

$$C_{mix} = \sum_{i=1}^{n} c_i \cdot \frac{EC_{50s}}{EC_{50i}} \quad (2)$$

where C_{mix} is the hypothetical sum of the concentrations of the compounds present in the mixture, c_i is the known concentration of compound i in the mixture and EC_{50s}/EC_{50i} is the relative potency of compound i according to the standard, s. This ratio is also termed the toxic equivalency factor.

The TEF method assumes that all the individual agents are full agonists with parallel dose-response curves differing only in their potency. This causes serious limitations for the applicability of this model and can lead to insufficient quality of the prediction. It is important to note that the whole concept of CA requires information about the dose of each component of the mixture that produces an effect equal to the toxicity effect of the whole mixture. Thus, it is very difficult to calculate the whole curve for a mixture that contains partial agonists for all the CA models. Eq. (1) cannot be used for description of mixture effects that exceed the maximum effect of the least potent component, because that effective dose cannot be defined (Scholze et al., 2014). This is a serious problem, because many environmental toxicants including estrogens or dioxin-like compounds are partial agonists of their respective receptors (Silva et al., 2007, 2011).

Many research studies approximate the maximum effect of a mixture as being equal to the maximum for the most potent compound (Grund et al., 2011; Johnson et al., 2013; Kunz and Fent, 2006; Payne et al., 2000; Rajapakse et al., 2002). However, it is obvious from other studies that the maximum mixture effect can also be substantially influenced by partial agonists (Scholze et al., 2014; Kunz and Fent, 2006; Rajapakse et al., 2001). This phenomenon should also be considered in a general model for the prediction of mixture effects. This is a serious problem in toxicology and only a few articles have attempted to examine this in detail (Geary, 2013; Howard and Webster, 2009).

Predicting the expected effects of a mixture of agents is an essential point of reference for the assessment of combination effects. At the present time, there is still no model that would be able to predict the whole dose-response curve of mixtures containing compounds with various potencies towards a receptor (EC_{50}), the slopes of their curves and their maximum observed effects.

Our study describes the development of a new predictive general model with verification using estrogenic compounds assayed with yeast recombinant and human cell line screens. This new model can be used to estimate the biological activity of mixtures comprising full and partial agonists and compounds with different slopes of their curves. This new approach assumes that every estrogenic compound acts towards the receptor according to a logistic function (Eq. (3)).

$$E = MAX + \frac{MIN - MAX}{1 + \left(\frac{c}{EC_{50}}\right)^{p}} \quad (3)$$

where E is the toxic effect, MIN and MAX represent the minimum and maximum of the curve, c is the concentration of the agent, EC_{50} is the concentration that gives half-maximal response represented by the inflection point and p is the slope parameter of the curve. All of these parameters and only these are the initial values for calculation of our model prediction. This logistic function is simply a different expression of the Hill function commonly used and recommended as regression in a dose-response relationship (Jenkinson et al., 1995). The equations for the Hill and logistic functions with minimum set to zero are equivalent. Our model also follows the principle of the CA model but does not use Eq. (1) as the initial basis for the derived equations. We call it the Full Logistic Model (FLM) since FLM uses all four parameters of the logistic curve without any simplification. The accuracy of predictions of mixture effects derived from this model were then compared with those calculated by the TEF and generalized concentration addition (GCA) approaches (Howard and Webster, 2009). The accuracy of the predictions was evaluated by comparison with the combination effects observed experimentally.

We employed the standardized yeast reporter gene assay to measure the effects of single compounds and their combinations including various ratios in the mixtures. This assay had the advantage that it monitors only activation or inactivation of the estrogen receptor without any other metabolic transformations or other adverse effects on the test organism. As a second test to verify our model, we employed the CXCL12 assay, which measured altered secretion of CXCL12/SDF1 by the T47D cell line. Secretion of this protein is regulated by activation of an estrogenic receptor and it was therefore an additional useful tool to measure the estrogenic response of our samples.

2. Materials and methods

2.1. Chemicals

Natural estrogen 17β-estradiol (E2; ≥98%) was used as a standard compound in all the calculations of mixtures. Other estrogenic compounds used in the tests were synthetic estrogen 17α-ethynylestradiol (EE2; ≥98%), bisphenol A (BPA; ≥99%) and the insecticide methoxychlor (MET; HPLC 98,7%). All the chemicals were purchased from Sigma-Aldrich (Prague, Czech Republic).

2.2. Yeast assay

The yeast assay uses the genetically transformed strains of *Saccharomyces cerevisiae* (BMAEREluc/ERα and BMA64luc), described in detail in the article by Leskinen et al., (2005). Stock solutions of the estrogenic compounds were dissolved in 30% (v/w) dimethyl sulfoxide (DMSO; ≥99.9%, Sigma-Aldrich, Prague, Czech Republic). The stock solutions for the mixtures with selected ratios of concentrations of the estrogens were prepared using solutions of the individual compounds. Serial dilutions of each of the constituents were also performed in 30% DMSO. E2 was used as a positive control in each experiment. The stock solutions were diluted 11 times with media containing the respective yeast strain so that the concentration of DMSO in the medium did not exceed 3% (Ezechiáš et al., 2012). The growing yeast strain was incubated with the tested compounds at 30 °C for 2.5 h. The culture was then shaken briefly and D-luciferin was added to the medium and immediately measured using a Lumino-M90a luminometer (ZD Dolní Újezd, Czech Republic). All the measurements were performed in triplicate.

2.3. CXCL12 assay

The human T47D breast carcinoma cell line was kindly provided by Dr. Truksa, Academy of Sciences of the Czech Republic. The CXCL12/SDF1 test was used as a second estrogenic test to quantify the estrogenic activity of the compounds alone and of their mixtures. The altered secretion of the stromal cell-derived factor 1 (SDF-1) was measured by the enzyme-linked immunosorbent assay – ELISA. This assay was carried out with stock solutions of each estrogenic compound dissolved in ethanol. Serial dilutions of each constituent were also performed in ethanol. T47D cells were routinely maintained in RPMI medium (Invitrogen, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine

serum, FBS (Invitrogen, Life Technologies, Carlsbad, CA, USA) and antibiotics (Invitrogen, Life Technologies, Carlsbad, CA, USA) at 37 °C in 5% CO_2. The assay was performed according to Habauzit et al. (2010). The cells were maintained in RPMI phenol red-free (Invitrogen, Life Technologies, Carlsbad, CA, USA), supplemented with 5% charcoal-stripped fetal bovine serum. For all the experiments, T47D cells were plated in 24-well plates with 7×10^4 cells/well in phenol red-free RPMI with 5% charcoal-stripped FBS. The tested compounds at various concentrations (see the results section) were added to wells so that the concentration of ethanol in the cell cultures did not exceeded 0.1%. All the tests were performed in tetraplicate. The cells were incubated for 2 days at 37 °C in 5% CO_2, after which culture supernatants of each repetition were pooled together and immediately measured by ELISA. The ELISA test was performed with a Quantikine kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.4. Prediction model

The concentrations of the individual compounds present in the mixture were recalculated according to the relative potencies of these compounds (Eqs. (4) and (5)).

$$TEQ_i = c_i \cdot \frac{EC_{50s}}{EC_{50i}} \tag{4}$$

$$TTEQ = \sum_{i=1}^{n} TEQ_i \tag{5}$$

TTEQ is the total toxic equivalent (or estrogenic equivalent) of a mixture that, in fact, represents the sum of the individual toxic equivalents (TEQ_i) of the components of the mixture. c_i is the concentration of compound i in the mixture and EC_{50s}/EC_{50i} is the relative potency of compounds i according to the respective standard representing the toxic equivalent factor. In our case, the natural ligand estradiol was used as the standard. Then we assume a theoretical concentration C_i^{hyp} of compound i that exhibits the same TTEQ according its specific potency represented by EC_{50s}/EC_{50i}.

$$TTEQ = C_i^{hyp} \cdot \left(\frac{EC_{50s}}{EC_{50i}}\right) \tag{6}$$

Substituting the value of C_i^{hyp} into Eq. (3) of the logistic function yields the hypothetical curves of the toxic (estrogenic) effect for each compound i that has its respective slope and the maximum of the original dose-response curve of the individual compound; however, the inflection points of these resulting curves are already equal (Eq. (7)) and represent the final EC_{50} of the mixture.

$$E_i^{hyp} = MAX_i + \frac{MIN_i - MAX_i}{1 + \left(\frac{C_i^{hyp}}{EC_{50i}}\right)^{p_i}} \tag{7}$$

The parameters MIN_i, MAX_i, p_i and EC_{50i} in Eq. (7) represent the values of compound i acting alone. E_i^{hyp} is the hypothetical effect of compound i, MIN_i and MAX_i are the minimum and maximum observed effects, respectively; p_i is the slope parameter and EC_{50i} is the inflection point of the curve. C_i^{hyp} is the hypothetical concentration obtained form Eq. (6).

The final effect of the mixture can then be calculated as the sum of the E_i^{hyp} values corrected by the respective relative toxic equivalents TEQ_i/TTEQ of each the component in the mixture (Eq. (8)).

$$E_{mix} = \sum_{i=1}^{n} \left(E_i^{hyp} \cdot \frac{TEQ_i}{TTEQ}\right) \tag{8}$$

In this equation, E_{mix} denotes the final effect of the calculated mixture and E_i^{hyp} is the hypothetical effect of compound i obtained from Eq. (7). TEQ_i represents the individual toxic equivalent of mixture component i. TEQ_i and TTEQ are values derived from Eqs. (4) and (5). Then, for example, substituting Eqs. (4)–(7) into Eq. (8) for a binary mixture results in:

$$E_{mix} = \left(MAX_1 + \frac{MIN_1 - MAX_1}{1 + \left(\frac{\left(\left(c_1 \cdot \frac{EC_{50s}}{EC_{50_1}}\right) + \left(c_2 \cdot \frac{EC_{50s}}{EC_{50_2}}\right)\right)}{\frac{\left(\frac{EC_{50s}}{EC_{50_1}}\right)}{EC_{50_1}}}\right)^{p_1}}\right) \cdot \left(\frac{\left(\frac{EC_{50s}}{EC_{50_1}}\right) \cdot c_1}{\left(\left(\frac{EC_{50s}}{EC_{50_1}}\right) \cdot c_1\right) + \left(\left(\frac{EC_{50s}}{EC_{50_2}}\right) \cdot c_2\right)}\right) + \left(MAX_2 + \frac{MIN_2 - MAX_2}{1 + \left(\frac{\left(\left(c_2 \cdot \frac{EC_{50s}}{EC_{50_2}}\right) + \left(c_1 \cdot \frac{EC_{50s}}{EC_{50_1}}\right)\right)}{\frac{\left(\frac{EC_{50s}}{EC_{50_2}}\right)}{EC_{50_2}}}\right)^{p_2}}\right) \cdot \left(\frac{\left(\frac{EC_{50s}}{EC_{50_2}}\right) \cdot c_2}{\left(\left(\frac{EC_{50s}}{EC_{50_2}}\right) \cdot c_2\right) + \left(\left(\frac{EC_{50s}}{EC_{50_1}}\right) \cdot c_1\right)}\right) \tag{9}$$

Eq. (9) can be simplified *via* elimination of EC_{50s} and the resulting version corresponds to Eq. (10) of FLM:

$$E_{mix} = \left(MAX_1 + \frac{MIN_1 - MAX_1}{1 + \left(\frac{c_1}{EC_{50_1}} + \frac{c_2}{EC_{50_2}}\right)^{p_1}}\right) \cdot \left(\frac{\frac{c_1}{EC_{50_1}}}{\left(\frac{c_1}{EC_{50_1}}\right) + \left(\frac{c_2}{EC_{50_2}}\right)}\right) + \left(MAX_2 + \frac{MIN_2 - MAX_2}{1 + \left(\frac{c_2}{EC_{50_2}} + \frac{c_1}{EC_{50_1}}\right)^{p_2}}\right) \cdot \left(\frac{\frac{c_2}{EC_{50_2}}}{\left(\frac{c_2}{EC_{50_2}}\right) + \left(\frac{c_1}{EC_{50_1}}\right)}\right) \tag{10}$$

All the values in this Eq. (10) are described above and, in contrast to the TEF approach, the dose-response parameters of the standard are no longer required.

For comparison with FLM, predictions using the previously published model mixtures were also calculated for comparison with our experimental results. The prediction using the TEF model was performed according to Eq. (2), where the resulting calculated mixture concentration was used in the logistic function for standard 17β-estradiol. Prediction of the mixture effect by the GCA model was calculated according to Eq. (12) from the article of Howard and Webster (2009).

2.5. Model fitting and significance testing

All the dose-response curves for the individual compounds followed the logistic function represented by Eq. (3) with $R^2 \geq 0.98$ or $R^2 \geq 0.90$ for the yeast assay or CXCL12 assay, respectively. In order to test the fit of the model responses of the mixtures to the experimental data, we used the sum of the squared residuals (SSR), normalized goodness-of-fit statistics *i.e.* Nash–Sutcliffe Efficiency coefficient (NSE; Ritter and Muñoz-Carpena, 2013) and the nonparametric Mann-Whitney test, which was used, *e.g.*, by Howard et al. (2010).

Additionally, we applied a statistical test hypothesis to the goodness-of-fit model according to Ritter and Muñoz-Carpena (2013). The null hypothesis (H0) assumes that the NSE means from all our measured experiments are lower than the threshold NSE value below which the goodness-of-fit is not acceptable ($NSE < NSE_{threshold} = 0.65$) and the alternative hypothesis (H1) when it is acceptable ($NSE > NSE_{threshold} = 0.65$) (Ritter and Muñoz-Carpena, 2013). The statistical significance of all the results was tested at the $\alpha = 0.05$ level using the one-sample *t*-test.

All the statistics and plots were performed and created using OriginPro 8.5 software.

3. Results

3.1. Prediction by full logistic model (FLM)

Fig. 1A demonstrates the example of a combination of three chemicals with different dose-response curve parameters predicted by FLM model that leads to the final partial agonistic dose-response curve. Compounds 1 and 2 are both full agonists with the same EC_{50}, differing only in the slope parameters. Compound 3 is a partial agonist, all of whose parameters are different. Due to the lower potency of the partial agonist in this case, the final mixture contains a 10 times higher concentration of this chemical. If the molar ratio of the components were 1:1:1, the two full agonists

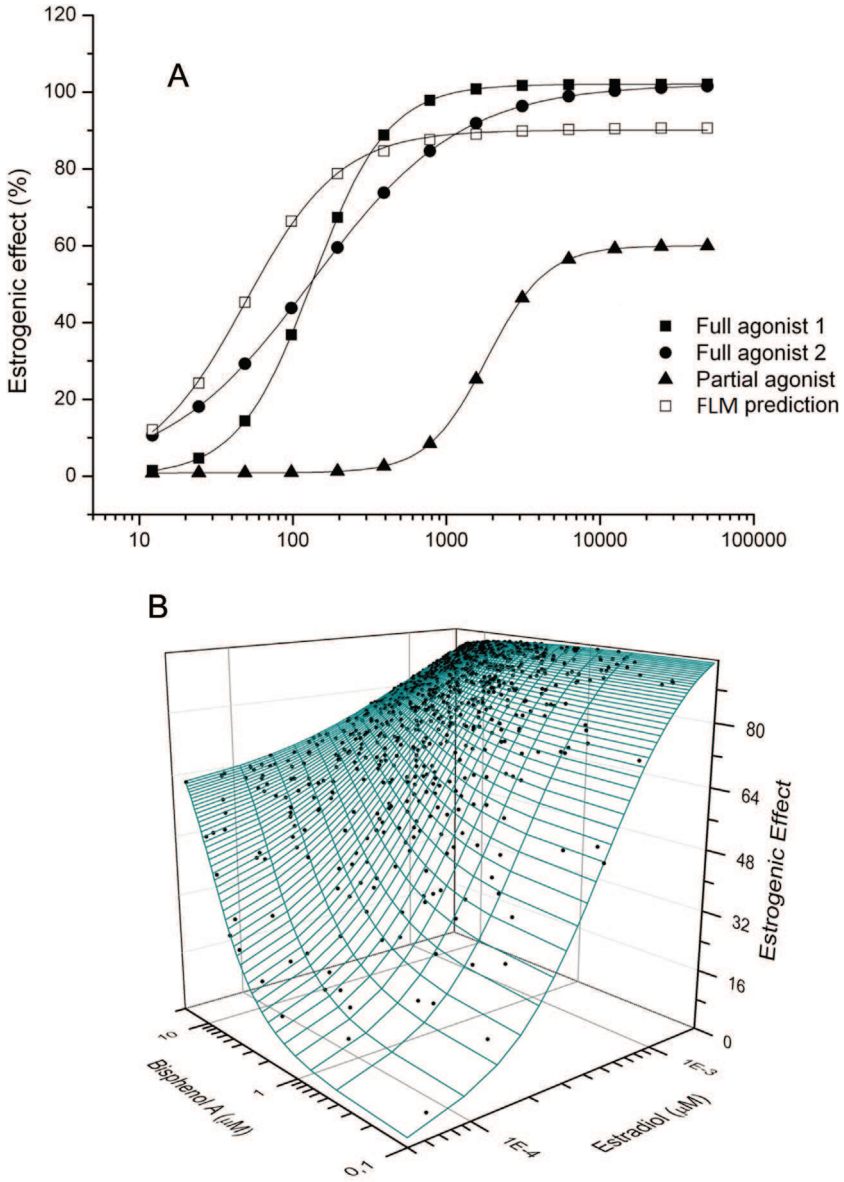

Fig. 1. A—An example of the FLM predicted curve of a hypothetical mixture of full and partial agonists. Each agonist is represented by its own effect towards the estrogenic receptor. The hypothetical mixture contains 50 mM each of agonist 1 and agonist 2 together with 500 mM of a partial agonist. B—The estrogenic response surface for a mixture of 17β-estradiol and bisphenol A computed by the FLM method. Each the point on the surface represents the estrogenic effect of a randomly generated combination of the estrogens.

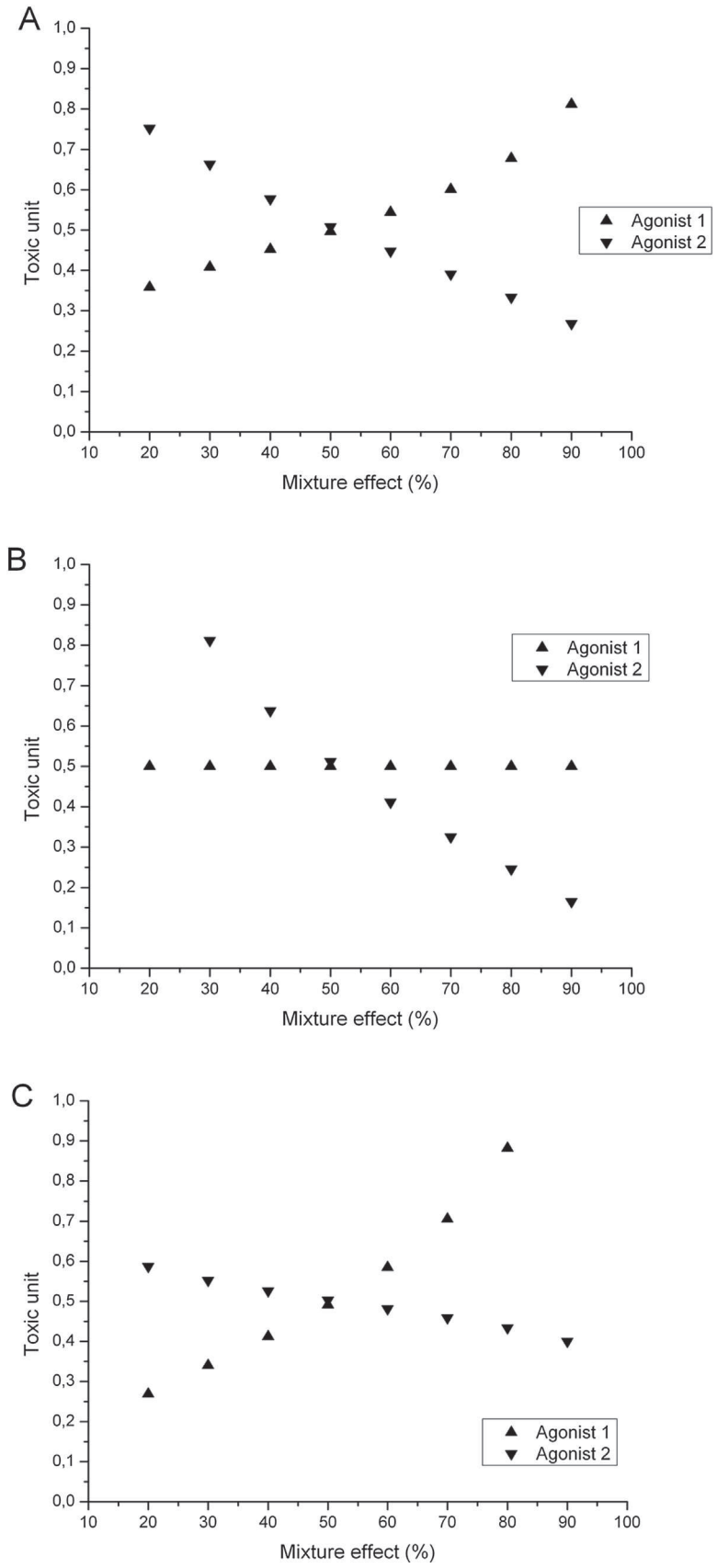

Fig. 2. Example of the toxic unit of each compound in the mixture according to the CA concept predicted by the FLM, TEF and GCA approaches. Toxic units calculated by FLM are shown in figure A, by TEF in figure B and by GCA in figure C. The data represent a mixture of two hypothetical agonists, when agonist 1 (▲) has a higher slope parameter of the curve than agonist 2 (▼). The other parameters of their curves (maximum, minimum and EC_{50}) are the same.

would almost balance out any contribution of the partial agonist in this mixture and the final mixture curve would not exhibit partial agonism.

Fig. 2 represents the behavior of the toxic units in a mixture consisting of two hypothetical estrogens possessing the same parameters but with different slope parameters p (1.78 and 0.9). The toxic unit was calculated using Eq. (1), where the toxic effect was set as an initial value and concentration ED_i was calculated with the respective logistic function of the individual compounds. The concentration d_i of compounds i in the mixture was calculated using the FLM, TEF and GCA predictions (Fig. 2A, B and C, respectively). The predicted dose-response curve of each model was created with selected concentrations (ratio 1:1) of these hypothetical compounds and a number of dilutions that exhibit the chosen mixture effect were evaluated. The individual concentrations d_i were then calculated from the initial concentrations and the dilutions. It is worth noting that the sum of the toxic units is approximately 1 throughout the whole concentration interval when evaluated using the FLM approach, in contrast to the other models. This demonstrates that our model can evaluate the effects of compounds with different slopes and also that the predictions are still in agreement with the concept of concentration addition where the sum of the toxic units is expected to be 1.

The other models (TEF and GCA) do not correspond to this assumption and the toxic units for one compound do not change or change only slightly (Fig. 2B and C). The sums of the toxic units

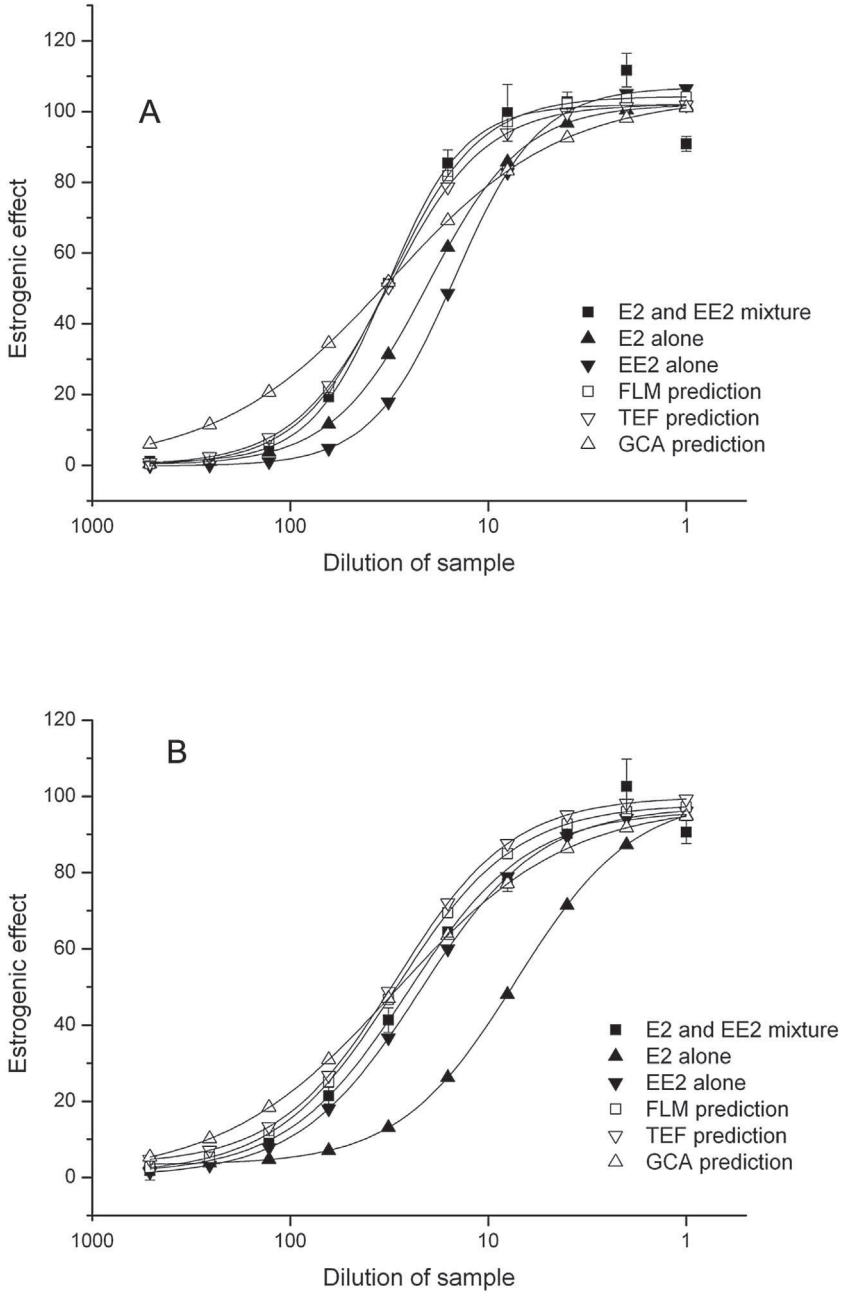

Fig. 3. A—Recombinant yeast assay. Estrogenic activity of a mixture (■) containing 91.78 nM of 17β-estradiol (E2) and 84.34 nM of 17α-ethynylestradiol (EE2). B—T47D–CXCL12 assay. Estrogenic activity of a mixture (■) containing 146.85 nM of 17β-estradiol (E2) and 134.95 nM of 17α-ethynylestradiol (EE2). FLM predicted curves of the mixtures (□) and estrogenic activities of samples containing only the respective concentrations of E2 without addition of EE2 (▲) and curves for the respective concentrations of EE2 without addition of E2 (▼) are depicted. TEF (▽) and GCA (△) represent the predicted curves using the toxic equivalency factor and the generalized concentration addition approaches, respectively. Y error bars represent the standard deviations of the experimental data.

from the TEF and GCA predictions are equal to 1 (and therefore follow the principle of CA) only when the mixture effect is 50%.

Another example of the dose-effect relationship of two chemicals in a mixture is illustrated by Fig. 1B. The dose response parameters of two real estrogens (E2 and bisphenol A) are utilized and the mixture effect was theoretically predicted using FLM with 990 random concentration combinations of these compounds. The results are depicted as the response surface of the final effect of the mixture. Bisphenol A acts as a partial agonist using our assays and thus the left side of the surface cannot reach the maximum

Table 1
Characteristics of the experimentaly obtained and predicted dose-response curves. EC_{50} is expressed as a dilution factor (folds of dilution) of the original mixture. MAX and p represent the maximum and the slope parameter p of the curve, respectively. SSR is the sum of the squares of residuals obtained after comparison of the experimental and the respective predicted data. NSE is a descriptive statistic demonstrating goodness-of-fit of the model (see the Materials and methods section).

Type of assay	Dose response characteristic	Experimental data	FLM	TEF	GCA
E2 (91.78 nM) + EE2 (84.34 nM)	EC50	32.26	31.53	31.53	31.53
yeast assay	Max	102.01	104.29	102.08	104.33
Fig. 3A	SSR		388.42	436.93	1599.66
	NSE		0.986	0.983	0.922
	slope	2.24	1.91	1.79	1.00
E2 (146.85 nM) + EE2 (134.95 nM)	EC50	25.67	29.52	29.52	29.52
CXCL12 assay	Max	96.19	97.99	100.00	98.00
Fig. 3B	SSR		228.72	398.92	395.34
	NSE		0.984	0.971	0.972
	slope	1.42	1.43	1.47	1.00
E2 (36.71 nM) + BPA (1095.10 μM)	EC50	14.23	18.40	20.23	18.40
yeast assay	Max	87.98	86.65	102.08	86.74
Fig. 4A	SSR		313.92	1974.20	1081.60
	NSE		0.977	0.856	0.921
	slope	2.34	2.04	1.79	1.00
E2 (73.43 nM) + BPA (4380.39 μM)	EC50	12.67	12.04	13.34	13.34
CXCL12 assay	Max	88.17	85.04	100.00	82.59
Fig. 4B	SSR		184.74	691.05	480.49
	NSE		0.984	0.939	0.957
	slope	1.30	1.33	1.47	1.00
E2 (458.92 nM) + MET (23144.80 μM)	EC50	72.30	53.17	53.23	53.22
CXCL12 assay	Max	76.21	72.46	100.00	72.52
Fig. 5A	SSR		679.01	2600.44	1313.28
	NSE		0.901	0.623	0.809
	slope	2.38	1.77	1.90	1.00
E2 (45.89 nM) + MET (23144.80 μM)	EC50	26.25	29.45	29.45	29.45
CXCL12 assay	Max	51.64	50.31	100.00	50.35
Fig. 5B	SSR		179.76	12720.63	192.50
	NSE		0.956	−2.099	0.953
	slope	1.34	1.58	1.90	1.00
E2 (367.13 nM) + BPA (1752.16 nM)	EC50	35.15	43.42	43.49	43.49
yeast assay	Max	96.80	93.78	100.00	93.93
Fig. 6A	SSR		177.21	236.70	723.66
	NSE		0.987	0.982	0.945
	slope	1.63	1.40	1.33	1.00
E2 (29.37 nM) + BPA (4380.39 μM)	EC50	28.15	25.26	25.26	25.26
yeast assay	Max	64.70	73.75	100.00	73.86
Fig. 6B	SSR		226.51	3994.35	358.26
	NSE		0.970	0.478	0.953
	slope	2.47	1.77	1.33	1.00
E2 (18.36 nM) + BPA (6570.59 μM)	EC50	27.53	35.47	35.48	35.48
yeast assay	Max	70.86	72.04	100.00	72.09
Fig. 6C	SSR		198.99	4169.54	603.87
	NSE		0.977	0.522	0.931
	slope	2.06	1.82	1.33	1.00
E2 (77.10 nM) + BPA (17521.57 μM)	EC50	56.47	37.67	38.12	38.12
CXCL12 assay	Max	78.18	88.46	100.00	89.97
Fig. 6D	SSR		440.12	2174.16	360.97
	NSE		0.944	0.725	0.954
	slope	1.67	0.93	1.90	1.00
E2 (77.10 nM) + BPA (5887.25 μM)	EC50	25.00	17.53	17.37	17.37
CXCL12 assay	Max	85.41	88.63	100.00	91.55
Fig. 6E	SSR		478.10	1175.09	465.64
	NSE		0.953	0.885	0.954
	slope	1.70	1.08	1.90	1.00
E2 (183.57 nM) + BPA (4818.43 μM)	EC50	34.82	32.06	32.32	32.32
+ MET (9642.70 μM)	Max	76.01	75.66	100.00	77.43
CXCL12 assay	SSR		358.69	3740.10	299.47
Fig. 7A	NSE		0.952	0.505	0.960
	slope	1.08	1.39	1.90	1.00
E2 (30.59 nM) + BPA (4818.43 μM)	EC50	19.05	30.03	30.22	30.22
+ MET (15429.87 μM)	Max	64.00	62.36	100.00	63.76
CXCL12 assay	SSR		292.29	7660.76	254.52
Fig. 7B	NSE		0.933	−0.744	0.942
	slope	1.15	1.20	1.90	1.00

response of E2. This 3D chart shows how the maximum mixture effect could vary according to the ratio of the two compounds.

3.2. Model and experimental data fitting with full estrogen agonists

The results of the first experiments demonstrate the estrogenic effect of a mixture containing E2 and EE2 (Fig. 3A and B). In fact, the results show the estrogenic effect determined using the recombinant yeast and CXCL12 assays when two compounds (E2 and EE2) were used at approximately the same concentrations with full agonistic activity toward the estrogenic receptor. The dose-response curves were predicted using FLM and also using TEF and GCA models. The characteristics of all the dose-response curves including the experimental data are shown in Table 1. In these cases, we obtained good predictions of EC_{50} with all three prediction approaches. Slightly worse results were recorded regarding the shape of the GCA predicted curve in the case of the recombinant yeast assay (Fig. 3A).

An attempt was made to analyze the curve fit using the nonparametric Mann-Whitney test; however, the predictions did not significantly differ from the experimentally measured curve ($P > 0.05$) in any case. The Mann-Whitney test also did not show significant differences in any of the other experiments and therefore the data are not presented and discussed further. Consequently, we employed the NSE and SSR tests and, e.g., the sums of the squares of the residuals document that the best fit was obtained using the FLM method yielding the lowest SSR (Table 1).

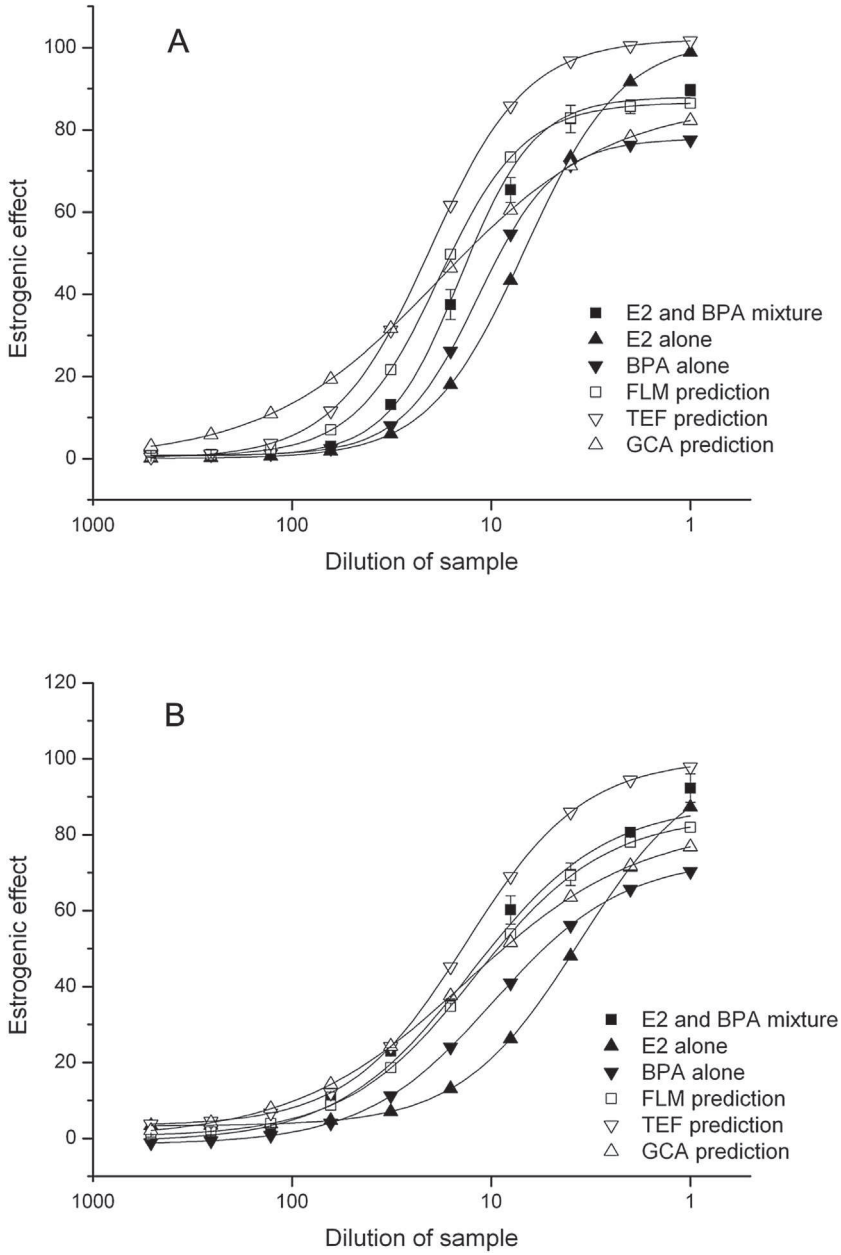

Fig. 4. A—Recombinant yeast assay. Estrogenic activity of a mixture (■) containing 36.71 nM of 17β-estradiol (E2) and 1095.10 μM of bisphenol A (BPA). B—T47D–CXCL12 assay. Estrogenic activity of a mixture (■) containing 73.43 nM of 17β-estradiol (E2) and 4380.39 μM of bisphenol (BPA). FLM predicted curves of the mixtures (□) and estrogenic activities of samples containing only the respective concentrations of E2 without addition of BPA (▲) and curves for the respective concentrations of BPA without addition of E2 (▼) are depicted. TEF (▽) and GCA (△) represent the predicted curves using the toxic equivalency factor and the generalized concentration addition approaches, respectively. Y error bars represent the standard deviations of the experimental data.

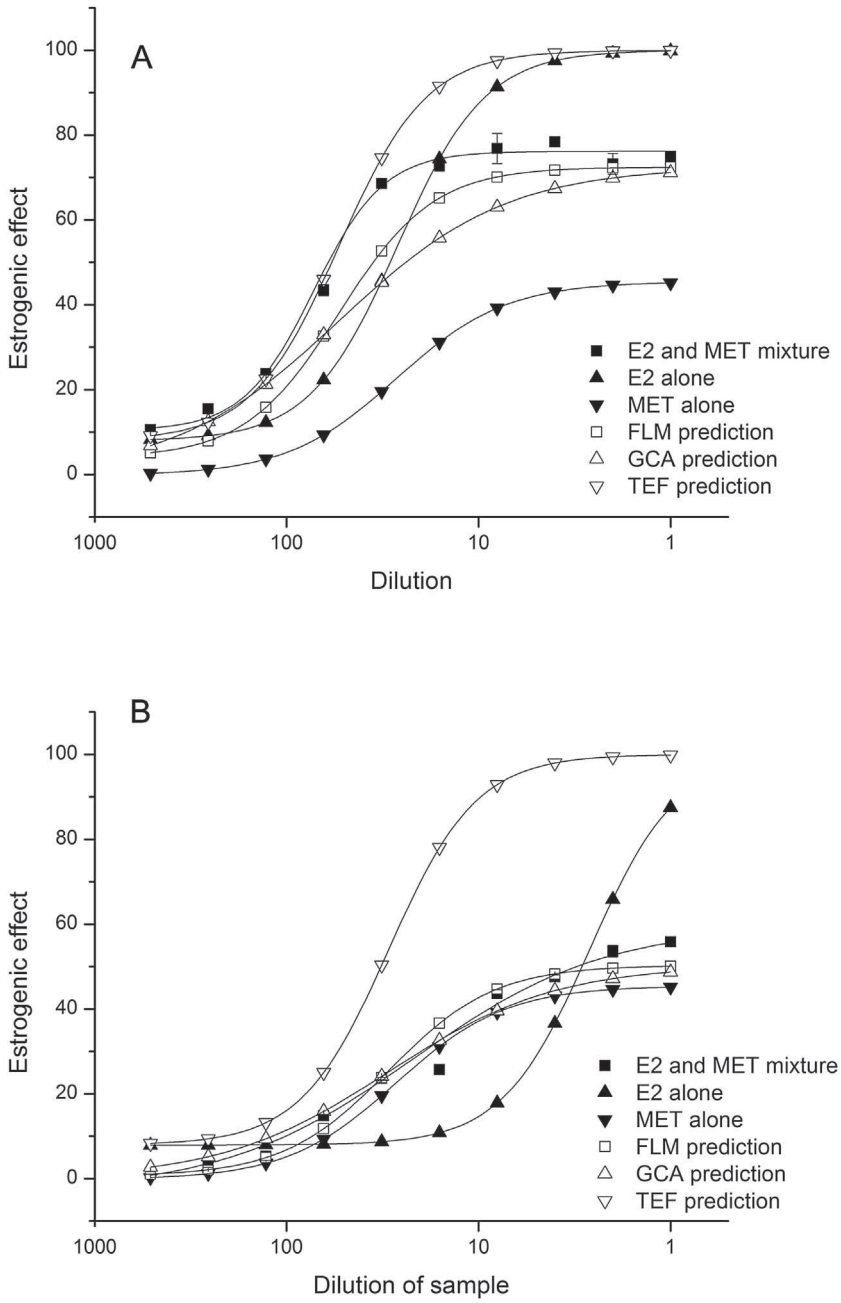

Fig. 5. A—T47D–CXCL12 assay. Estrogenic activity of a mixture (■) containing 458.92 nM of 17β-estradiol (E2) and 23144.80 μM of methoxychlor (MET) (potency ratio 1:1). B—T47D–CXCL12 assay. Estrogenic activity of a mixture (■) containing 45.89 nM of 17β-estradiol (E2) and 23144.80 μM of methoxychlor (MET) (potency ratio 1:10). FLM predicted curves of the mixtures (□) and estrogenic activities of samples containing only the respective concentrations of E2 without addition of MET (▲) and curves for the respective concentrations of MET without addition of E2 (▼) are depicted. TEF (▽) and GCA (△) represent the predicted curves using the toxic equivalency factor and the generalized concentration addition approaches, respectively. Y error bars represent the standard deviations of the experimental data.

3.3. Model and fitting experimental data with full and partial agonists

In another experiment, a full agonist (E2) was combined with a partial agonist (BPA) and both the assays were again employed. BPA exhibited partial agonist behavior (see Fig. 4A and B) in both cases. Approximately equipotent amounts of the compounds represented by a molar concentration ratio of 1:29,831 and 1:59,654 (E2:BPA) were employed in the recombinant yeast and CXCL12 assays, respectively. The results of both the assays together with the model predictions are shown in Figs. 4A and B and the characteristics of all the dose-response curves including the experimental data are listed in Table 1. Overall, the best results were obtained using the FLM approach. This fact is well documented by the lowest SSR and highest NSE values, again exhibiting the best fit of this model to the experimental data.

The second partial agonist used in our study was methoxychlor (MET), which reached a maximum of 45.5% of the full agonist estradiol (see Fig. 5A and B) measured by the CXCL12 assay. These experiments could not be performed with the yeast assay due to the low solubility of MET in 30% DMSO and thus we analyzed the mixtures with this chemical using only the CXCL12 assay. Fig. 5A depicts approximately an equipotent mixture of E2 and MET represented by a molar concentration ratio of 1:50,433 (E2:MET) and Fig. 5B shows the same mixture but with a molar concentration ratio of 1:504,334 (E2:MET). In both cases, our model predicted that the character of the resulting curves would be

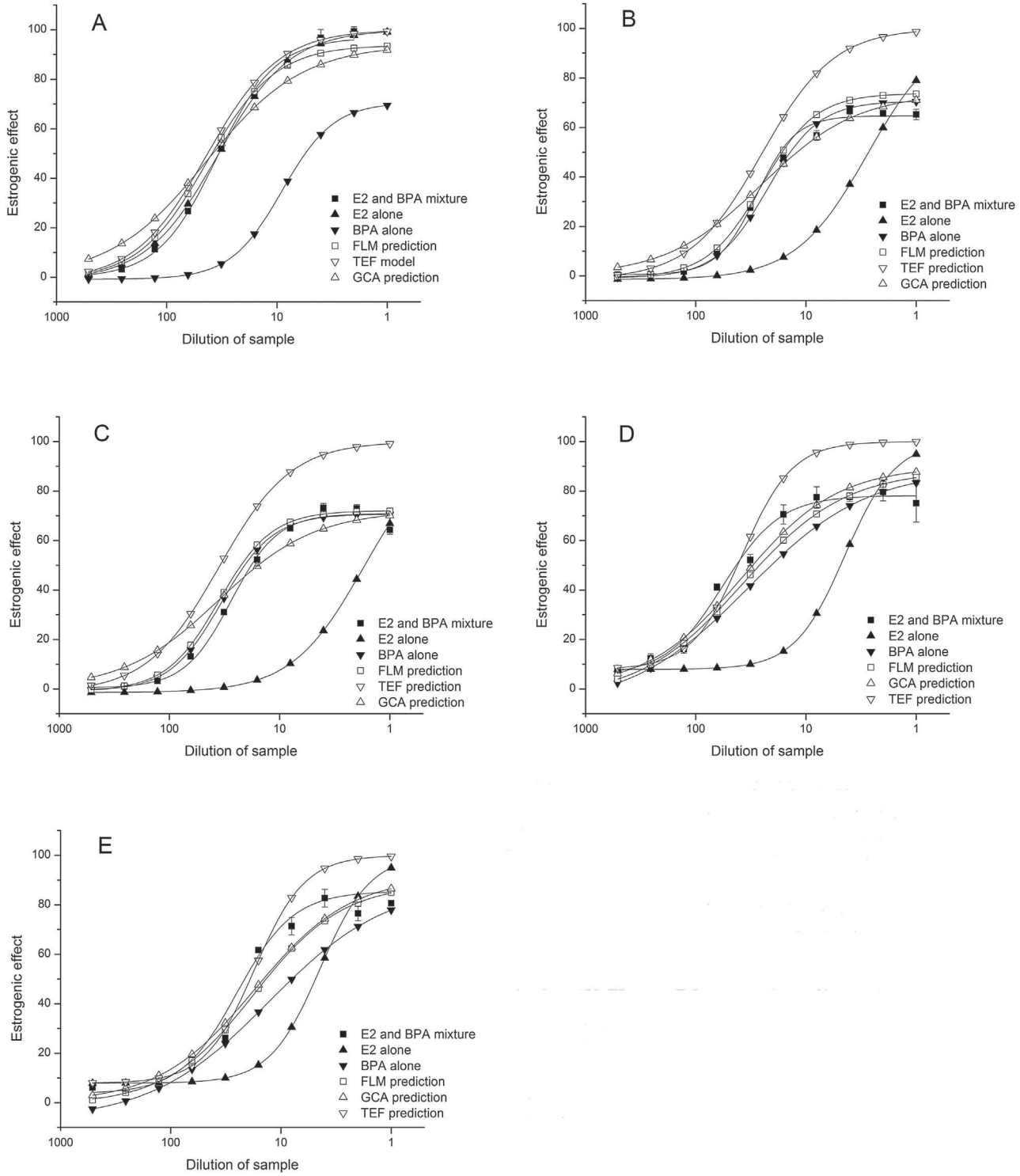

Fig. 6. A—Recombinant yeast assay. Estrogenic activity of a mixture (■) containing 367.13 nM of 17β-estradiol (E2) and 1752.16 nM of bisphenol A (BPA) (potency ratio 4:1). B—Recombinant yeast assay. Estrogenic activity of a mixture (■) containing 29.37 nM of 17β-estradiol (E2) and 4380.39 μM of bisphenol (BPA) (potency ratio 1:8). C—Recombinant yeast assay. Estrogenic activity of a mixture (■) containing 18.36 nM of 17β-estradiol (E2) and 6570.59 μM of bisphenol (BPA) (potency ratio 1:20). D—T47D–CXCL12 assay. Estrogenic activity of a mixture (■) containing 77.10 nM of 17β-estradiol (E2) and 17521.57 μM of bisphenol (BPA) (potency ratio 1:9). E—T47D–CXCL12 assay. Estrogenic activity of a mixture (■) containing 77.10 nM of 17β-estradiol (E2) and 5887.25 μM of bisphenol (BPA) (potency ratio 1:3). FLM predicted curves of the mixtures (□) and estrogenic activities of samples containing only the respective concentrations of E2 without addition of BPA (▲) and curves for the respective concentrations of BPA without addition of E2 (▼) are provided. TEF (▽) and GCA (△) represent the predicted curves using the toxic equivalency factor and the generalized concentration addition approaches, respectively. Y error bars represent the standard deviations of the experimental data.

partially agonistic due to the presence of MET in contrast with E2. Also in this case, the FLM approach predicted generally better results than TEF and GCA. The characteristics of all the dose-response curves including the experimental data are given in Table 1. The results in Fig. 5A and B also document that, as expected, the maximal mixture effect varies according to the ratio of full and partial agonists. In order to verify FLM at different partial agonist levels, we analyzed several mixtures with E2 and BPA by

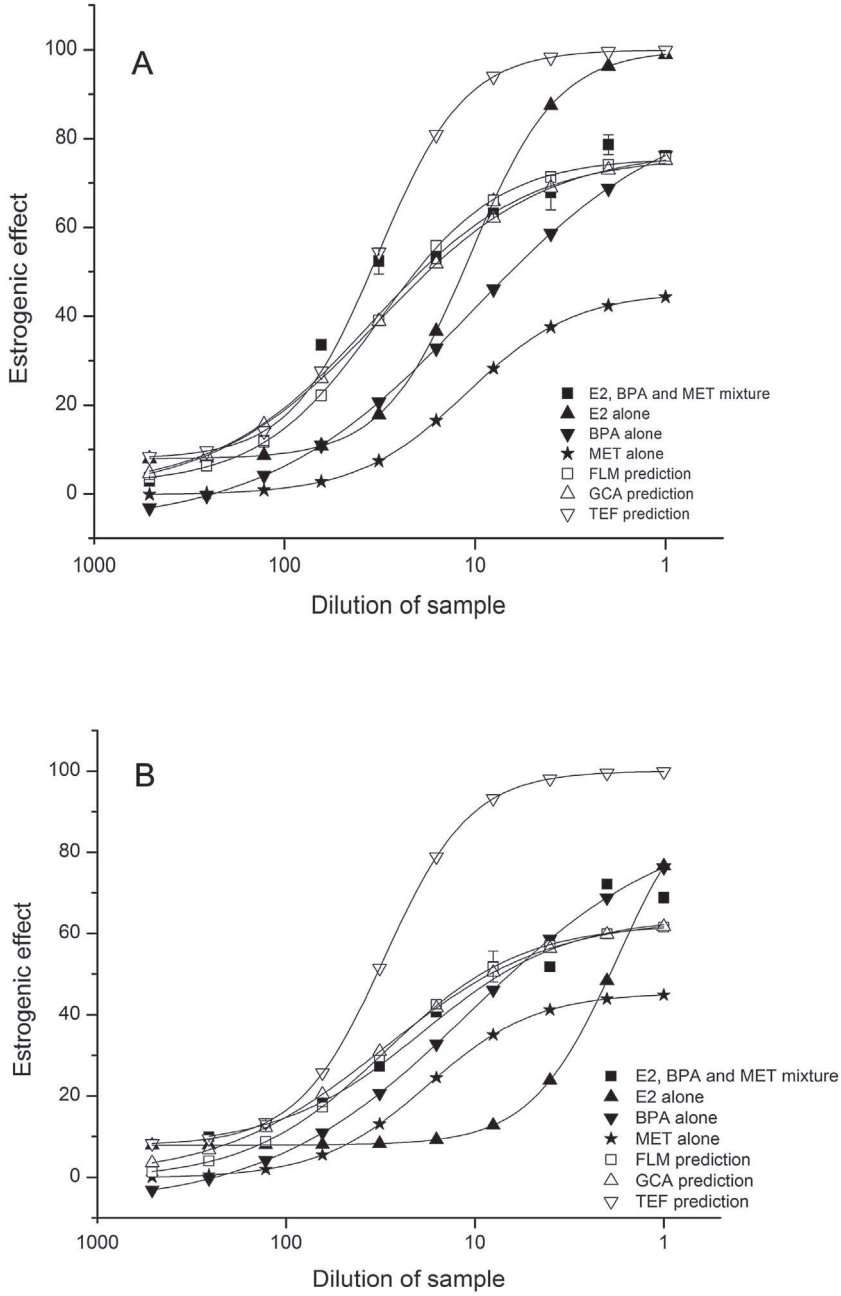

Fig. 7. A—T47D–CXCL12 assay. Estrogenic activity of a mixture (■) containing 183.57 nM of 17β-estradiol (E2), 4818.43 μM of bisphenol A (BPA) and 9642.70 μM of methoxychlor (MET) (potency ratio 1:1:1). B—T47D–CXCL12 assay. Estrogenic activity of a mixture (■) containing 30.59 nM of 17β-estradiol (E2), 4818.43 μM of bisphenol (BPA) and 15429.87 μM of methoxychlor (MET) (potency ratio 1:6:10). FLM predicted curves of the mixtures (□), estrogenic activities of samples containing only the respective concentrations of E2 without addition of BPA or MET (▲), curves for the respective concentrations of BPA without addition of E2 or MET (▼) and curves for the respective concentrations of MET without addition of E2 or BPA (★) are provided. TEF (▽) and GCA (△) represent the predicted curves using the toxic equivalency factor and the generalized concentration addition approaches, respectively. Y error bars represent the standard deviations of the experimental data.

both the estrogenic assays. The results are displayed in Fig. 6. It can be seen that the response of the mixture with a higher proportion of the full agonist (Fig. 6A; potency ratio 4:1, E2:BPA, respectively) yields a dose-response curve similar to the full agonist dose-response curve and therefore the results correspond to the TEF prediction. On the other hand, an increased content of the partial agonist (potency ratio 1:8 or 1:20, E2:BPA, respectively) caused substantially lowering of the resulting dose-response curve maximum (see Fig. 6B and C). Based on the statistical test, the FLM model predicted the final dose-response curve better than the other approaches in most cases (Table 1). The only exceptions were recorded for the CXCL12 assay with the employed potency ratios of 1:3 and 1:9 of E2:BPA, respectively, when GCA reached slightly better statistical indicators. These results are shown in Figs. 6E and D and Table 1.

3.4. Model and fitting experimental data with ternary mixtures

In addition, ternary mixtures with a full agonist (E2) and two partial agonists (BPA and MET) were measured with the CXCL12 assay in another experiment. Approximately equipotent amounts of the compounds represented by a molar concentration ratio of 1:26,246:52,530 (E2:BPA:MET) were employed and the results are displayed in Fig. 7A. We also measured a second ternary mixture

(Fig. 7B) with a molar concentration ratio of 1:157,493:504,335 (E2:BPA:MET). The potency ratio for this second mixture is approximately 1:6:10, see Fig. 7B. In both cases, the FLM and GCA models provided very good predictions of the mixture dose-response curves and fitted the experimental data well when GCA reached slightly better results (Table 1).

3.5. Goodness-of-fit statistics of the models

Apart from evaluation of the individual mixtures using SSR and the NSE statistics, the null hypothesis was tested assuming that the NSE means from all our measured experiments are lower than the threshold NSE (0.65). This hypothesis was rejected for the FLM and GCA models. Both models had an NSE value significantly higher than the value set as the threshold of acceptance. On the other hand, the TEF model failed in this test ($P = 0.803$). A higher value of the threshold (0.9) was also tested, yielding very good goodness-of-fit (Ritter and Muñoz-Carpena, 2013). Similarly, both the FLM and GCA models provided significantly higher values than the threshold, attaining P-values of 6.55×10^{-7} and 0.0038, respectively. The obtained P-value of the FLM model demonstrates the excellent and general prediction capabilities of this approach.

4. Discussion

The literature contains two major concepts for dealing with the phenomenon of mixture effects: CA and Independent joint action (IA). Independent joint action, which was first defined by Bliss (1939), is based on the idea of dissimilar action of the components of the mixture. Dissimilar means the primary interaction of toxicants with different molecular target sites and the triggering of a common toxicological endpoint (Bliss, 1939; Hadrup et al., 2013). We focused only on mixtures with a similar mode of action, as well as the CA concept; consequently, only the models derived from CA are discussed below. The TEF approach in the form of Equation (2) and its derivatives are common forms of the CA concept, which are widely employed for laboratory-prepared mixtures or environmental samples (Grund et al., 2011; Hadrup et al., 2013; Johnson et al., 2013; Kunz and Fent, 2006; Payne et al., 2000; Rajapakse et al., 2004).

TEF estimates an equivalent dose (concentration) using the original concentrations and relative potencies of the individual compounds, where the calculated concentration of the mixture is substituted into the equation of the dose-response curve of the respective standard (e.g. E2 for estrogens). Therefore, such an approach is valid only if the maxima and the slopes are equal for all the constituents of the mixture. The presence of a partial agonist substantially limits the TEF approach (Geary, 2013; Howard et al., 2010; Safe, 1997). The model also ignores that the curves for the individual compounds have different slopes. This is demonstrated by Fig. 2B. Due to the fact that the TEF model calculates predictions only with the EC_{50} values of the respective individual compounds, the correct results are obtained only for a mixture effect of 50%, i.e. resulting in EC_{50}. Predicting the effects of two compounds with different slopes by the TEF approach often leads to different outcomes and predicts false synergism (the sum of the toxic units < 1) or antagonism (the sum of the toxic units > 1) as described in detail by Geary (2013). Because of these limitations, the TEF model can be successfully used, for example, only in cases with full estrogenic agonists including natural hormones when the resulting curve will be similar to the E2 standard. Consequently, we obtained very similar predictions of the TEF and FLM models only in Figs. 3A and B, where two full agonists were used with similar individual parameters, and in Fig. 6A where the mixture contained a substantially higher portion of full agonist. In all the other cases where the partial agonist in the mixtures influenced the overall effect, the TEF model could not provide acceptable predictions (Figs. 4–7).

Howard and Webster (2009) described their own solution to the problem *via* the GCA model. This concept uses the inverse Hill function with slope parameter $p = 1$. They obtained a form of the concentration addition by substituting the inverse function for ED_i into Eq. (1). In their article, they demonstrated that, in order to extend the applicability of CA to partial agonists, it is necessary to have a model that produces nonparallel isoboles with a slope of any sign. In contrast to GCA, the TEF approach produces only parallel linear isoboles (Geary, 2013; Howard and Webster 2009; Tallarida, 2006).

This is an important fact because the resulting partially agonistic curve can be obtained only with a model where the isoboles can switch from negative to positive slopes. Such a consideration requires the theoretical assumption that, above the concentration of the partial agonist maximum effect, the partial agonist cannot increment the effect; however, it still competes with a full agonist for the receptor sites. The idea that partial agonists competitively antagonize full agonists is not new. Howard and Webster (2009) considered this concept in their GCA. Other authors also described the idea that partial agonists should have antagonist properties (Ariëns, 1964; Goldstein et al., 1974).

The main disadvantage of GCA is that the model uses the Hill function with only fixed slope parameter $p = 1$. Howard and Webster performed this simplification because use of the same concept, but with the Hill function where $p \neq 1$, leads to more complicated equations from which the toxic effect cannot be derived. Consequently, the resulting dose-response curve yielded by GCA always has a fixed slope of 1. This is visible in Figs. 3–7, where the GCA model predicted well the value of EC_{50} and the maximum effect but ignored the overall shape of the curves. Howard et al. (2010) documented that the dose response function of GCA with $p = 1$ is suitable for binary mixtures of dioxins and other agonists of the aryl hydrocarbon receptor; however, for instance, estrogens usually exhibit curves with a wide range of slope parameters (Silva et al., 2007; Kunz and Fent, 2006).

A more recent approach to calculating the effects of mixtures containing partial agonists is described in an article by Scholze et al. (2014). They used the toxic unit extrapolation method, where the toxic unit for a partial agonist in the mixture is fixed at a certain level corresponding to its saturation. This requires a decision about the numerical value of the partial agonist toxic unit and, as a consequence, it is necessary to choose an effect level where extrapolation of the curve is begun (Scholze et al., 2014). The main problem with the toxic unit extrapolation method is that the extrapolated curve of a partial agonist is considered to be fully agonistic. This completely overlooks the fact that the partial agonist could act as a weak antagonist in the mixture, as was discussed for the GCA approach above. This antagonistic effect of a partial agonist therefore cannot lead to the maximum predicted mixture effect of 100%. Mixtures which contain partial agonists often provide dose-response curves that are also partially agonistic. This could be seen in Figs. 4–7 in our study and also in other studies (Hadrup et al., 2013; Kunz and Fent, 2006; Rajapakse et al., 2001) including Scholze et al. (2014).

Our FLM model, in contrast to the TEF and GCA approaches, calculates all the parameters of the final dose response curve and predicts curves that could be partially agonistic, as can be seen in Fig. 1A. The fact that the sum of toxic units for full agonists with different slopes in Fig. 2A equals approximately 1 indicates that the data are still in agreement with the isobole CA concept and, in this case, the final predicted mixture effect is iso-additive (Loewe and Muischnek, 1926; Loewe, 1953). The FLM as well as GCA isoboles can switch from negative to positive slopes when the effect level exceeds the maximum effect of a partial agonist. Consequently, our

model and also GCA are capable of making predictions for mixtures containing a partial agonist. The calculated maximum effect found by our model is similar to the value obtained by GCA, so that the two models consider the antagonistic properties of partial agonists at a similar level.

The results displayed in Table 1 document that all the models yielded similar EC_{50} values; however, the models differ in other parameters of the dose-response curves and in most cases FLM exhibited better prediction of the experimental data as documented by the generally lowest SSR values, the highest NSE and the statistical test using the NSE values from all the experiments (see the Results section). In contrast to the previously published TEF and GCA models, our model does not require any prerequisites for correct prediction such as the parallel dose-response curves with the same maximum for the TEF approach or dose-response curves with a fixed slope equal to 1 for the GCA approach.

In contrast to the previously described GCA and TEF models, FLM utilizes all the necessary parameters of the original constituent dose-response curves (EC_{50}, slopes, maximum) to construct the final dose-response curve of the resulting mixture. Consequently, FLM also considers partial agonists and their effect on the final curve, in contrast to TEF; in addition, the slope of the final curve can be obtained from FLM, in contrast to GCA and TEF. The new model was verified using two estrogenic assays where, in both cases, FLM yielded generally better results than GCA and TEF. Only *in vitro* reporter assays that do not take into account *in vivo* metabolism and pharmacokinetics were employed; thus it can be assumed that the measured effect is caused only by activation or inactivation of the receptor itself. Using these two different *in vitro* methods provided further evidence for the validity of our model.

5. Conclusions

FLM can calculate the mixture effects from the individual curves with any shape or maximum and therefore we can conclude that this is the most complex model for mixtures of compounds with similar modes of action developed to date. This new method can also be applied to other classes of drugs (even therapeutic) or environmental pollutants and has the potential to improve the analysis and risk assessment of mixtures. Thus, the FLM model could serve as a new standard tool to calculate the mixture effect of toxic or biologically active substances and to assess contributions of individual pollutants in mixtures to the total toxicity of samples.

Conflict of interest

None.

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Receptor partial agonists and method to express receptor partial activation with a respect of novel models of mixture toxicology

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ABSTRACT
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Introduction
Human bodies are daily exposed by a various chemicals, drugs and environmental pollutants which may cause an adverse effect on human health. Commonly accepted concept how chemical or drug causes an effect is the receptor theory. Receptor theory stated that chemicals (agonists) acts through the macro-molecules (receptors) and interacts with the specific biding site on the receptor. This interaction may cause an activation or inactivation of respective receptor and consequent biochemical reactions. An agonist can be recognized pharmacologically either as a full agonist or as a partial agonist according to its capability to elicit the maximum response mediated by that specific type of receptor. Receptor full agonists, irrespective of their different receptor binding affinities, are all capable to cause a full maximum response. In contrast receptor partial agonists are unable to elicit a full maximum response regardless of their concentration[1]. This situation occurs when not enough of the receptor occupied by the agonist convert to an active form and remains inactive[2,3]. This situation in toxicology is described by a scheme:

$$(1)\ L + R \overset{K_1}{\Leftrightarrow} LR \overset{K_2}{\Leftrightarrow} LR^+$$

$$K_1 = \frac{[LR]}{[L] \cdot [R]}$$

$$K_2 = \frac{[LR^+]}{[LR]}$$

Where L is a ligand, R is a receptor, LR is a receptor-ligand complex and LR^+ is an active form of receptor ligand complex. K_1 and K_2 are microscopic equilibrium association constants. In contrast to a full agonist where receptor-ligand complex automatically convert to an active form, in case of partial agonism some amount of an inactive form (LR) should occur in equilibrium, but in our experiments we are usually not able to measure the concentrations of this inactive form. The macroscopic equilibrium constant of whole reaction is given by equation (2)[3].

$$(2)\ K = \frac{K_1 \cdot K_2}{1+K_2}$$

Where K is macroscopic equilibrium constant and K_1 and K_2 are microscopic equilibrium constants from scheme (1).

It seems that partial agonists which bind to a receptor have some portion that after binding remain inactive. So we can split the concentration of this compound to a two part. One part with a properties of a full agonist where every receptor-ligand complex automatically transform to an active form and the other one with properties of a competitive antagonist which compete for the same binding site, but after binding remains inactive. However, both parts should have the same affinity towards biding site of the receptor.
Recent studies from the field of mixture toxicology brings novel opportunities how the partial agonists could be utilized in mixtures[4,5]. These studies follow the concept of concentration addition and provided two novel mathematical models for calculation of mixture effects. We used equation (10) from the article of Ezechias and Cajthaml and the equation (12) from the article of Howard and Webster expressed in this study as an equation (3) and (4) respectively.

(3)

$$E_{mix} = \left(MAX_1 + \frac{MIN_1 - MAX_1}{1 + \left(\frac{c_1}{EC_{50_1}} + \frac{c_2}{EC_{50_2}} \right)^{p_1}} \right) \cdot \left(\frac{\frac{c_1}{EC_{50_1}}}{\frac{c_1}{EC_{50_1}} + \frac{c_2}{EC_{50_2}}} \right) + \left(MAX_2 + \frac{MIN_2 - MAX_2}{1 + \left(\frac{c_2}{EC_{50_2}} + \frac{c_1}{EC_{50_1}} \right)^{p_2}} \right) \cdot \left(\frac{\frac{c_2}{EC_{50_2}}}{\frac{c_2}{EC_{50_2}} + \frac{c_1}{EC_{50_1}}} \right)$$

(4) $$E_{mix} = \frac{MAX_1 \cdot \frac{c_1}{EC_{50_1}} + MAX_2 \cdot \frac{c_2}{EC_{50_2}}}{1 + \frac{c_1}{EC_{50_1}} + \frac{c_2}{EC_{50_2}}}$$

These new models allow us to use dose-response curves that are partial agonists (MAX < MAX of full agonist) and also incorporate compounds with no agonistic effect (MAX = 0). This is substantial advantage against previously used models which cannot calculate mixture effect for compounds with no agonistic effect on their own. We demonstrate that these novel approaches to calculate mixture effect could utilize the antagonistic properties of one compound and therefore result in a form of partial agonistic dose-response curve[4].
In this study, we apply these new methods of mixture toxicology to the paradigm of partial agonists and utilize the antagonistic part of partial agonist as a separate compound with zero effect. This also brings the general hypothesis that the competitive antagonist is nothing more than agonist with no effect. This concept may significantly enhance our understanding of receptor theory and the mode of action of receptor partial agonists. This allows us to derive the expressions how the microscopic and macroscopic equilibrium constants could be calculated and also obtained from the measured partial agonistic dose-response curve. As a result of this, we derive a simple mathematical tool how the dose-response curves of drugs could be compared according their affinities and efficacies.

Results and discussion

Dose-response curves for agonists in the presence of antagonist

The assumption that the some portion of molecules of partial agonists which bound to a receptor remains inactive could be mathematically described as a combination of two compounds where one compound have zero effect on its own ($MAX_2 = 0$). The added concentration of partial agonist could be split into c_{ago} and c_{antago} where c_{ago} represents concentration of molecules which after binding to a receptor convert it to an active form (LR^+) and $c_{anatago}$ represents concentration of molecules which after binding to a receptor remains inactive (LR). After the substituting these parameters for full agonist and competitive antagonist into the equations (3) and (4) and plotted the results in the concentration-response diagram we obtain partial agonistic dose-response curve. The maximal effect of this curve is dependent on the ratio of concentrations c_{ago} and c_{antago}. On figure 1a are few examples of these resulted curves with different rations of c_{ago}:c_{antago}. Full agonistic curve resulted only when $c_{antago} = 0$; changing the ratio in favor of c_{antago} resulted in diminishing the curve maximal effect (fig. 1a).
The assumption that combination of agonist with a competitive antagonist resulted in the form of partial agonistic dose-response curve is not new. Zhu also show that this combination resulted in a form of partial agonistic dose-response curve. Only difference is, that he assume hybrid compound which covalently link a molecule of agonist with an antagonist[1]. This hybrid compound resulted in a situation where the molar concentration of agonistic and antagonistic part are always equal but difference with their affinities towards receptor. In our situation we considered partial agonist as two compounds with a same affinity but differ with their concentrations, but the result is the same (you may compare fig. 1a with a fig. 1a of Zhu [1]).
From the models of mixture toxicity (equations (3) and (4)) we derive the equation describing the relation between maximal effect of the resulted partial agonistic dose-response curve and the ratio of c_{ago} and c_{antago}.

(5) $MAX_{partial} = MAX_{avail} \cdot \frac{c_{ago}}{c_{ago}+c_{antago}}$

Or synonymosly

(6) $MAX_{partial} = MAX_{avail} - MAX_{avail} \cdot \frac{c_{antago}}{c_{ago}+c_{antago}}$

Where $MAX_{partial}$ is the maximal effect of partial agonists dose-response curve, MAX_{avail} is the maximal effect that could be achieved in the test and c_{ago} and c_{antago} are the concentrations of agonistic and antagonistic fraction of the compound respectively. For further details about derivation of this equation for maximal effect please see Supplementary materials. If we set the value of MAX_{avail} on 100% then the expression of $MAX_{partial}$ will be in percentage of the maximal achievable effect in the test.
Another important issue came from the fig. 1a is, that all resulted curves has the same value of inflex point (EC_{50}). The EC_{50} is defined as a dose or concentration that produces 50% of the maximal response to that drug ($MAX_{partial}$, but not MAX_{avail})[3], therefore in every dose-response curve EC_{50} value is the inflex point of the curve even for the partial agonists. Antagonistic moiety in this situation does not shift the curve to right or left. The EC_{50} value is strictly dependent on the sum of the concentrations c_{ago} and c_{antago}. Therefore if we keep the sum of the concentrations constant, the EC_{50} of resulted dose-response curve will also be constant. This is a different situation that the commonly accepted theory that if we add fixed concentration of antagonist, the curve for agonist will shift to the right. In our situation we have mixture and the increase of one part automatically cause an increase of the second. From this point we could assume that the parameter EC_{50} remains as a factor of affinity of compound towards receptor and it is not affected if the compound`s molecule binding on the receptor actually trigger the effect or not.
In toxic tests we usually measure only effect triggered by the active receptors (LR^+) but this does not mean that inactive receptors (LR) are not presented. According to the results above, antagonistic part of partial agonist is always bound to the receptor with the same affinity and serve as a complement of partial agonistic dose-response curve to the full agonistic. Figure 1b describe this situation. With the assumption of absence of spare receptors, the toxic effect (%) has the same scale as receptor occupancy (%) so the ligand will always achieve the full receptor occupancy (100% of total receptor R_T), however in this case we would only measure maximal effect of this compound 83.3% (the rest of receptors are occupied but inactive). This situation may also result in a form of partial agonistic dose-response curve with very small or almost none maximum effect. In that case, majority of receptors are occupied by ligand but remains inactive (LR). The overall shape of the curve remains the same as for full agonist (curve A in fig. 1a), it has its EC_{50} and slope, but we cannot measure any effect. This compound is rather considered as a competitive antagonist, because it only compete for the receptor binding sides but has no effect on its own. Due to this, in mixture models we could treat the competitive antagonists as the agonists (partial agonists) with a zero maximal effect.

Gaddum`s equation
Gaddum`s equation is a well known mathematical formulation of interaction of agonist with a competitive antagonist. In his work Gaddum simple applied the law of mass in the interaction of ligands with receptors and provided followed mathematical formula (equations (7) and (8))[6,7].

(7) $P = \frac{A}{A+K_A\left(1+\frac{B}{K_B}\right)}$

Or with a different notation:

(8) $\frac{A}{K_A} = \frac{P}{1-P}\left(1+\frac{B}{K_B}\right)$

Where the P is the proportion of receptors occupied by the agonist, A and B are the concentrations of agonist and antagonist respectively and the K_A and K_B are the equilibrium dissociation constants of agonist and antagonist respectively. For practical purposes values EC_{50} of compounds A and B can be taken as a constants K_A and K_B respectively.
Gaddum`s equation referred only to proportion of occupied receptors, not to receptor response. Under the assumption that there are no spare receptors in the test (every receptor could be involved and all receptors should be occupied in order to produce the maximal effect) we could express proportion of occupied receptor P as a proportion of toxic effect (E/Emax).

Gaddum`s equation as stated applied the simple law of mass action, that means that it does not take into account that compounds may have various slopes of their curves. If we assume that the concentration of antagonist is zero in equation 8 (B = 0), we obtain exactly the Hill function with the hill coefficient (slope parameter) equal 1. This is understandable, because the first signs that the curves may vary in their slopes came years after the Gaddum formulate this law.
After the application of hypothesis that the competitive antagonist is just an agonist with no effect, we could transform the equation (3) for a two agonist into the Gaddum`s equation. For further details about the derivation please see respective section in Supplementary materials. Transformation of the equation (4) for a second model is even easier, because this model combine only the compounds with a slope parameter 1, and it was also previously demonstrated[5].
The fact that the Gaddum`s equation could be derived from the models for combination of agonist brings some evidence that our hypothesis about competitive antagonists is valid. This demonstration that the equation for agonist-agonist interaction could be transformed into the equation for agonist-antagonist brings little revolution, because it link together two historically major paths in computational pharmacology. One path focusing on interaction of agonist with an antagonist is represented by a work of J.H. Gaddum, H.O. Schild, A.J. Clark, D. Colquhoun and the others[6-13]. The second path is focusing on the practical combination of two agonists with common outcome and calculation of this additive effect; this is represented by a work of S. Loewe, S.H. Safe, and the others[14-18]. Both discussed models in this article represents the evolution of the second path and only the assumption, that the antagonist is nothing more than agonist with no effect, which leads to a Gaddum`s equation, represents nice connection between these two approaches.

Efficacy and relation with equilibrium constant
The concept that drugs may have varying capacities to initiate response and consequently occupy different proportions of the receptors when producing equal responses was firstly described by Stephenson. This property was referred to as the efficacy of the drug[19]. Efficacy came as a new parameter for the compounds, independent from the affinity. In his work Stephenson pointed out that efficacy is key ability for partial agonists and also refer that these compounds possess properties intermediate between agonist and antagonist. Antagonists have zero efficacy[19]. In mathematical form efficacy is defined by a simple function S = ey, where S is the parameter of stimulus of drug, e is the efficacy and y is the proportion of occupied receptors. Stephenson pointed out, that his parameter stimulus is not equal as measured response (R) and remain the relation between these values as a "black box" where R = f(S). On the other hand, he stated that efficacy can have any positive value and adopt the convention that the stimulus is equal 1 for a 50% response. It is clear, that if efficacy could achieve any positive value with no theoretical limit, the stimulus can also have any positive value; however the toxic response measured in the test is usually limited to certain maximal level.
Some misunderstandings and ambiguity in the term efficacy were caused after another term intrinsic efficacy was introduced by Furchgott[20,21]. He used the same notation as Stephenson and defined intrinsic efficacy by function S = ε[LR], where S is the stimulus, ε is the intrinsic efficacy and [LR] is the concentration of receptor-ligand complex. In his articles Furchgott, same as Stephenson, remained the relationship between stimulus and measured response as a black box (R = f(S)), so the practical usage for the parameter efficacy still missed.
For the first reaction in scheme (1), after applying law of mass action and Hill-Langmuir equation, we can express the fraction of occupied receptors as an equation (9)[2,3].

$$(9)\ \frac{[LR]}{[R_T]} = \frac{[L]}{[L]+\frac{1}{K_1}} = y$$

Where y is the fraction of occupied receptors, [L] is the concentration of ligand and K_1 is the equilibrium association constant.
After application the same law of mass action on second reaction in scheme (1) we can express the fraction of active occupied receptors (LR^+) as an equation (10). For detailed derivation of this equation please see respective section in Supplementary materials.

$$(10)\ \frac{[LR^+]}{[R_T]} = \frac{y}{y+\frac{1}{K_2}} = \frac{K_2 \cdot y}{K_2 \cdot y + 1}$$

Under the assumption that there are no spare receptors, the fraction of active occupied receptors is equal as a fraction of measured effect (E/E_{avail}). This fraction of measured effect is analogous to the response (R) used by Stephenson and Furchgott. This is also stated in the article of Furchgott and Bursztyn (1967). From this point we could combine together with the Stephenson`s expression.

(11) $R = \frac{E}{E_{avail}} = \frac{[LR^+]}{[R_T]} = \frac{K_2 \cdot y}{K_2 \cdot y + 1} = f(S) = f(ey)$

Where E is the measured effect and E_{avail} is the potential maximal effect in the test. All other parameters are described above.
From this point it is clear that equilibrium association constant K_2 serve as a e, therefore efficacy (e) is nothing more than equilibrium constant for the second reaction in scheme (1).

(12) $e = K_2 = \frac{[LR^+]}{[LR]}$

The whole function for the parameter stimulus (referred black box) can be written as equation (13).

(13) $R = \frac{S}{S+1}$

This expression of compound efficacy is much better for practical use. It has calculable mathematical form and contains no reference to the number of total receptors, or proportion of occupied receptors. This expression of efficacy also correspond to the convention suggested by Stephenson that a drug which occupies all the receptors (y = 1) to produce 50% effect of the true maximum (R = ½) has an efficacy equal 1 and also correspond to the adoption that stimulus is equal 1 for a 50% response.
Other authors also refer that the parameter efficacy correspond to the second reaction in scheme (1) and to the conversion of the receptor to its active form[11,22,23]. On the other hand, scientists use several methods to measure or express the efficacy of the drug, which cause the ambiguity in the definition of efficacy. This is reviewed in the article of Strange (2008) with several examples from the literature[24]. In this article we followed the first definition of efficacy by Stephenson and demonstrated, that it has strictly mathematical form related to the equilibrium constant. We suggest that this mathematical expression of efficacy (equation 12) should be preferred in literature. The same expression of efficacy is used in the work of Colquhoun (1987) where author also relate the efficacy with the equilibrium constant and provide similar equations as above[25].

Spare receptors
In this article we consider only assay conditions with the absence of spare receptors and in certain cases this assumption is crucial for mathematical formulation (for example in equation (11)). Concept of spare receptors (sometimes termed receptor reserve) was introduced by Furchgott (1966) according the experimental measurements where the chemical inactivation of some portion of receptors does not result in a decrease in maximal measured response of full agonist[20]. And at sufficient high degrees of receptor inactivation, the maximum response even to full agonists was finally reduced. Furchgott conclude that there have to be some portion of receptors in the test that is not necessary to obtain maximal response. This conclusion rise the question that measured response in the test is not strictly dependent on the concentration of receptors.
This concept of spare receptors is often associated with the efficacy by theory that agonist with higher efficacy than full agonist could occupy and also activate the spare receptors which lead to a shifting the dose-response curve to the left (and therefore measured lower EC_{50} value)[12,19]. This seems strange, because if the efficacy corresponds with the equilibrium constant as described above, the true full agonist should automatically convert receptor-ligand complex to its active form and no inactive form should appear in the equilibrium. That means that the true full agonist must have the maximal efficacy (limiting to infinity) and none other compound can achieve higher value. Explanation for this lays in the experimental design.
On figure 2 is the example of dose-response curves from the experiment where activation of only 1% of receptors are sufficient to produce maximal measured effect. Drug A in this situation can activate only 1% of receptor population and 99% remains inactive, but according to the observed effect we could consider this drug as a full agonist. But this drug is not the full agonist, it has efficacy only 0.01, but it seems to be full agonist due to the experimental conditions. In this situation it is easy to conclude that if only 1% of total receptors are sufficient to produce maximal effect, the rest 99% of receptors are spare (receptor reserve). Compounds B, C and D can activate even smaller portion of receptor population and due to this they have their maximal effect lower than A and are considered as partial agonists. On the other hand, drugs E, F, G and H have higher efficacy than A; with efficacy 0.015, 0.05, 0.25 and infinite respectively. Only the drug H is the true full agonist which could activate all the receptors. It is clear, that resulted does-response curves for compounds with a higher efficacy than A shifts to the left and observed EC_{50} values seems to be lower, because the experimental conditions "cut off" the true curve (dash lines) and we observe only modified curves (solid lines). It is important to note, that curves for drugs A-D and the dash curves for drugs E-H has the same inflex point, drugs has the same K_1 and therefore the same affinity towards receptor. They only differ with their efficacy. Increasing in efficacy does not

cause the increase of affinity towards receptor, because these parameters are separated from each other in principle. It only cause apparent shift of dose-response curve and lower observed EC_{50} value.
The influence of experimental design to the measured parameters of dose-response curves are discussed in some recent articles. Stott et al. discussed how the assay condition influence dose-response curve analysis and wrote: "By reducing the receptor population sufficiently enough so that even at full occupancy, the full agonist can no longer induce a maximal response, receptor reserve is eliminated. This moves the EC_{50} of the concentration-response curve closer to the K_1 value…" [26]. She also added that in case of partial agonist, full occupancy still generates a sub-maximal response. This is absolutely in agreement with our presented results and Fig. 2 and 1b. Zhu in his article also discussed the effect of spare receptors on the dose-response curves and provided the similar figure with "cut off" curves as Fig. 2. He stated that with increasing spare receptor populations, the apparent EC_{50} values of the curves become smaller[1]. But this is only apparent EC_{50} which does not correspond to the affinity of compound.
These effects of spare receptors may be explained by assay conditions. Many assays use tissues or cell lines which highly express the certain receptor which can lead to high levels of receptor reserve, whereby submaximal occupation of receptors can lead to maximal responses at downstream. That means that limiting factor for such assays is not the concentration of receptors, but the concentration of some other reactant in the following signaling cascade. Moving further downstream of the receptor cascade can lead to increased signal amplification, whereby low levels of receptor activation can still lead to maximal responses. This is well discussed in the article of Stott et al. (2016) where authors demonstrate that only changing the endpoint in signaling cascade of receptor binding assay may cause different outcome where one compound change from partial to full agonist[26]. A partial agonist in a receptor binding assay may easily appear as a full agonist using an endpoint further downstream despite the fact that the efficacy for the ligand–receptor combination remains the same[26]. In our extreme situation (Fig. 2), where activation of only 1% of receptors is sufficient for maximal measured effect, drug A may change from antagonist to a full agonist. If we plot the figure 2 with the same scale on left and right axis like on figure 1b, we would see, that drug A is the partial agonist with measured maximum of only 1% and the rest of the receptor occupancy (area between drug A and the full agonist drug H) is filled by inactive form of LR. In that case, drug A would rather be considered as a competitive antagonist than agonist.
Amplification of expressed receptors and increasing the concentration of receptors in the test may be useful for low efficacy ligands, which can be difficult to detect, but in order to obtain correct values from the dose-response curves it is necessary to measure at assay conditions with absence of spare receptors. This is the key factor of our derived equations and for the next section about the obtaining of equilibrium constant from the dose-response curves. We assume that this requirement about absence of spare receptors should also be considered in the development of some novel receptor binding assays.

Equilibrium constants and dose-response curves
If our measurement met the conditions of absence of spare receptors as described in previous section, we can derive the microscopic equilibrium constants from the measured dose-response curve. Another important requirement is that binding assay should be at equilibrium as it is discussed in the review of Hulme and Trevethick (2010). From the scheme (1), equilibrium constants for the first reaction can by written as:

$$(14)\ K_1 = \frac{[LR]}{[L]\cdot[R]}$$

In the situation when 50% of receptors are occupied, the molar concentration of receptor-ligand complex is equal as molar concentration of remaining receptors without ligand. Concentration of unbound ligand in this situation is equal to administered concentration (EC_{50}) minus the concentration of ligand that bound to the receptor, which is half of the total concentration of the receptors in the test ($[R_T]$). We can rewrite the equation (14) in the form of equation (15)[27].

$$(15)\ K_1 = \frac{1}{EC_{50} - \frac{[R_T]}{2}}$$

Some authors omits the parameter for the total receptors and assume measured EC_{50} from the curve equal as dissociation constant ($K_{1d} = EC_{50}$). This could be applied because concentration of ligand usually exceeds the concentration of receptors by more than magnitude. This is also one of the conditions suggested by Hulme and Trevethick which ideal receptor binding study should follow[27]. For example concentrations of natural ligand estradiol assayed on human cell line usually exceed the concentration of receptors more than 10^4 times. Despite the simplification, the use of the exact equation is still recommended for data analysis.
In the respective section above we demonstrated that the inflex point of measured curve is always constant regardless the dose-response curve is partial agonistic or not. In assay conditions with absence of spare receptors,

the efficacy of the drug does not affect the measured EC_{50}. That means we could determine the equilibrium constant even from partial agonist dose-response curve.
For a second reaction in scheme (1), equilibrium constant can be express as.

$$(16)\ K_2 = \frac{[LR^+]}{[LR]}$$

Where K_2 is the respective equilibrium constant, $[LR^+]$ is the concentration of active receptor-ligand complex and [LR] is the concentration of inactive receptor-ligand complex. For the full agonists, concentration [LR] is zero and therefore constant K_2 limits to infinity. We assume that the ratio of molar concentrations $[LR^+]/[LR]$ is equal to the ratio of c_{ago} / c_{antago} representing the concentrations of agonistic and antagonistic moiety in previous section. Therefore we can rearrange the equation (6) to obtain the expression how the value of K_2 could be calculated.

$$(17)\ K_2 = \frac{[LR^+]}{[LR]} = \frac{c_{ago}}{c_{antago}} = \frac{MAX_{avail}}{MAX_{avail} - MAX_{partial}} - 1 = \frac{MAX_{partial}}{MAX_{avail} - MAX_{partial}}$$

All variables are described above. According to the equation (17), the value of K_2 could be calculated only with the knowledge of observed maximal effect of curve and the maximal effect that could be achieved in the test. If the value of $MAX_{partial}$ will be half of the MAX_{avail} then resulted K_2 will be 1, which represents the same concentration of active and inactive form of receptor-ligand complex. This is still in agreement with Stephenson`s assumption that a drug which produce 50% effect of the maximum has an efficacy (K_2) equal 1.

Potency and compare of drugs
In literature there is still uncertainty with using the term potency. Many research uses the parameter EC_{50} (or K_{1d}) of compounds as potency, however EC_{50} is parameter only for affinity and drug potency should depends on both affinity and efficacy[3]. One of the solution is to describe the action of drug with macroscopic equilibrium constant K which combine both affinity (K_1) and efficacy (K_2). On figure 3a is an example of drugs A and B with respective EC_{50} 2.34 x 10^{-4} and 4.04 x 10^{-4}. If we compare the potency of these compounds only using values of EC_{50}, we get that drug B is 1.73 times more potent than drug A. However drug B is the partial agonist and cannot achieve the same maximum as A. On figure 3b is another example using compounds with same EC_{50} as before, but maximal effect for drug C is even lower. In this section we provide the simple method how the potency of partial agonist could be handle and compare to each other using equilibrium constant K.
For full agonist, macroscopic constant K is equal to K_1 ($K=K_1$), because K_2 limits to infinite and the whole reaction expressed by equations (1) and (2) is dependent only on K_1. In case of partial agonist, some inactive form of [LR] is presented at equilibrium and K_2 is not infinite so the overall K should be lower (equation 2). We propose to calculate value of EC_{50} which could achieve the partial agonist with certain K transformed to the full agonist with the same K. This is kind of correction of EC_{50} according to the maximal effect of the drug. Equation (18) explains this in general.

$$(18)\ \frac{1}{EC_{50cor} - \frac{[R_T]}{2}} = \frac{\left(\frac{1}{EC_{50} - \frac{[R_T]}{2}}\right) \cdot K_2}{1 + K_2}$$

This expression is just a combination of equations (2) and (15). We imagine dose-response curve that is full agonist and obtain the same macroscopic K as certain partial agonist. This hypothetical full agonist should have higher value of EC_{50} than original; here indexed as EC_{50cor}. Parameter EC_{50cor} is the corrected value of measured EC_{50} according to the efficacy of the drug.
Substituting the equation (17) into equation (18) and rearranging we could get.

$$(19)\ EC_{50cor} = \left(EC_{50} \cdot \frac{MAX_{avail}}{MAX_{partial}}\right) - \left[\frac{[R_T]}{2} \cdot \left(\frac{MAX_{avail}}{MAX_{partial}} - 1\right)\right]$$

From equation (19) it is clear that if we follow this method for the full agonist, which has the ratio of $MAX_{avail}/MAX_{partial}$ equal to 1, than the EC_{50cor} is identical to the observed EC_{50}. In case of partial agonism, portion of $MAX_{avail}/MAX_{partial}$ is greater than one, so resulted value will be higher than original EC_{50}. Second part of this equation with a concentration of total receptors follows the same, but when maximal effect is between 50-100% of MAX_{avail}, it is become even smaller. As stated before, measured value of EC_{50} in

experiment usually exceeds the $[R_T]$ by more than magnitude. Due to this, it is reasonable for practical uses omit the second bracket with concentration of total receptors and use only the equation (20).

$$(20)\ EC_{50cor} = \left(EC_{50} \cdot \frac{MAX_{avail}}{MAX_{partial}}\right)$$

This value of EC_{50cor} represents the potency of drug better than EC_{50}, so we suggest to use this corrected value when comparing two drugs. It is important to note, that this expression of potency require the measurement with no spare receptors, because presence of spare receptors affect the measured EC_{50} and $MAX_{partial}$ as it was described in respective section above. In previous example drugs B and C have the maximal effect 77.8 and 53 in Fig. 3a and 3b respectively. By using his approach with equation (20) we could write.

$$(21)\ \frac{EC_{50corA}}{EC_{50corB}} = \frac{EC_{50A} \cdot \frac{MAX_{avail}}{MAX_{partialA}}}{EC_{50B} \cdot \frac{MAX_{avail}}{MAX_{partialB}}} = \frac{\frac{EC_{50A}}{MAX_{partialA}}}{\frac{EC_{50B}}{MAX_{partialB}}}$$

As a result we obtain that drug B is 1.34 times more potent that drug A (Fig. 3a). This is lower value than previously reported 1.73 obtained by only comparison of EC_{50} value. On the other hand, despite the fact that drug C has the lower EC_{50} (higher affinity) than drug A (Fig. 3b), applying this approach resulted, that drug C has lower potency than drug A.
Equation (21) also demonstrated that in order to compare two agonists no value of maximal measureable effect in the test (MAX_{avail}) is required. For whole this approach it is necessary to compare dose-response curves of drugs obtained by the same assay (same tissue, receptor, conditions etc.).
Equation (21) represents a simple tool how the drug could be compared according their affinity and efficacy. Ehlert and collaborators in their work also provide a method for calculating the activity of an agonist which is mathematical identical as our equation (21) and termed it the IRA value (later RA_i)[28]. In the original mathematical formulation, the IRA value is derived only as a ratio of equieffective concentrations without adding any mathematical form of efficacy; also derivation of IRA value required crucial omitting of one parameter[29]. Besides the Ehlert`s work we derive the equation (21) using equilibrium constants for affinity and efficacy (K_1 and K_2), derived from the models of mixture toxicology. To avoid confusion, we propose to use the same term RA_i for this ratio. In previous articles RA_i value prove to be useful tool for comparison of various drugs and also for determining other effects on receptor-ligand interaction like ligand bias[26,28,30]. As mentioned above, the main advantage of this method is that the RA_i value can always be estimated even there is insufficient information about maximal response in the assay.

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Author contributions statement

M.E. conceived the idea and derived the equations, M.E. and T.C. wrote the manuscript.

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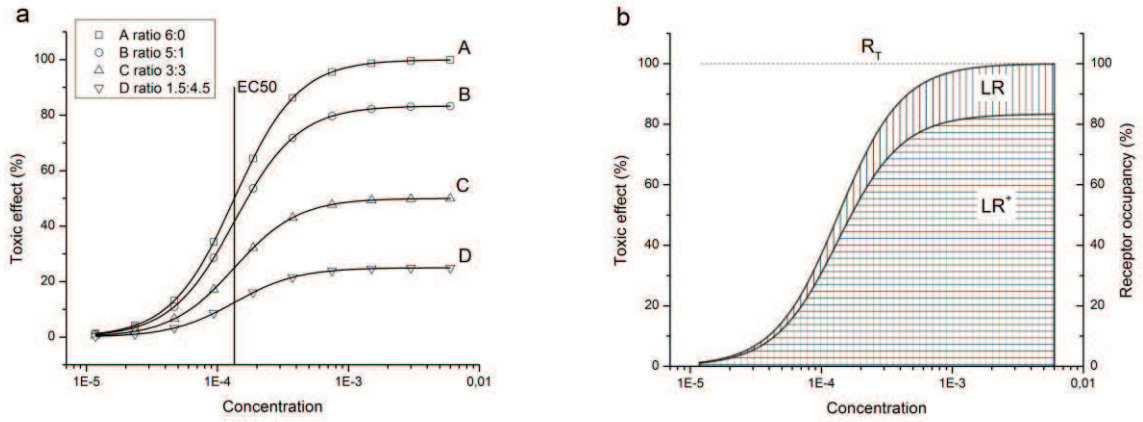

Figure 1. Dose-response curves in the presence of competitive antagonist. (a) The dose-response curves for agonist in the co-presence of competitive antagonist with the same affinity. Individual curves represents different concentration ratio of agonistic and antagonistic moieties and are 6:0; 5:1; 3:3; 1.5:4.5 for the curves A; B; C; D respectively. The x axis displayed the sum of concentrations of both part. (b) The dose-response curve of partial agonist which can activate only 83.3% of receptors. The rest of the receptors are also occupied by agonist but remain inactive (LR area) and represent complement of the curve to the full agonistic with the same inflex point.

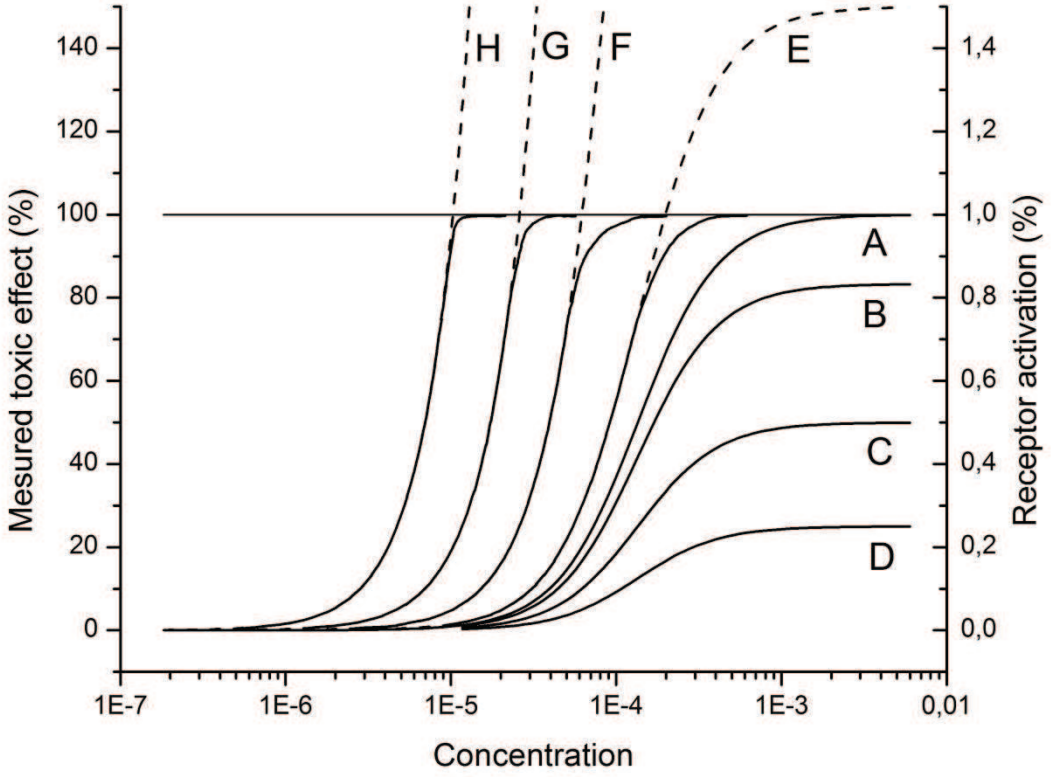

Figure 2. Presence of spare receptors and its effect on the measured dose-response curves. The dose-response curves for drugs with the same affinities (K_1) but different efficacies measured with a highly amplified test where activation of only 1% of receptors is sufficient to produce maximal measured effect. The efficacies for individual curves are 0.01; 0.0083; 0.005; 0.0025 for A; B; C; D respectively and 0.015; 0.05; 0.25; maximal for curves E; F; G; H respectively. The presence of spare receptors caused that the apparent EC_{50} value of the dose-response curve become smaller.

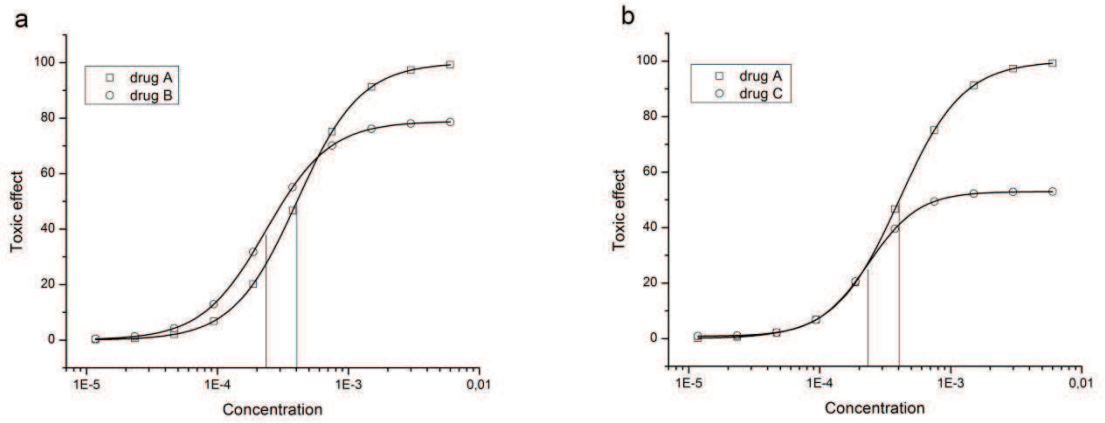

Figure 3. Comparison of dose-response curves according the potency. Example of dose-response curves for two drugs differing with the affinity and efficacy towards receptor. (a) Drug A is the full agonist with the EC_{50} value 4.04 x 10^{-4}; drug B is the partial agonist with the maximal effect of 78.9% and the EC_{50} value 2.34 x 10^{-4}. (b) Drug A is the full agonist with the EC_{50} value 4.04 x 10^{-4}; drug C is the partial agonist with the maximal effect of 53% and the EC_{50} value 2.34 x 10^{-4}.

Biodegradation of endocrine disruptors in urban wastewater using *Pleurotus ostreatus* bioreactor

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Abstract

The white rot fungus *Pleurotus ostreatus* HK 35, which is also an edible industrial mushroom commonly cultivated in farms, was tested in the degradation of typical representatives of endocrine disrupters (EDCs; bisphenol A, estrone, 17β-estradiol, estriol, 17α-ethinylestradiol, triclosan and 4-n-nonylphenol); its degradation efficiency under model laboratory conditions was greater than 90% within 12 days and better than that of another published strain *P. ostreatus* 3004. A spent mushroom substrate from a local farm was tested for its applicability in various batch and trickle-bed reactors in degrading EDCs in model fortified and real communal wastewater. The reactors were tested under various regimes including a pilot-scale trickle-bed reactor, which was finally tested at a wastewater treatment plant. The result revealed that the spent substrate is an efficient biodegradation agent, where the fungus was usually able to remove about 95% of EDCs together with suppression of the estrogenic activity of the sample. The results showed the fungus was able to operate in the presence of bacterial microflora in wastewater without any negative effects on the degradation abilities. Finally, a pilot-scale trickle-bed reactor was installed in a wastewater treatment plant and successfully operated for 10 days, where the bioreactor was able to remove more than 76% of EDCs present in the wastewater.

Keywords: wastewater treatment; bioreactor; endocrine disruptors; ligninolytic fungi; *Pleurotus ostreatus*

Introduction

Wastewater treatment plants (WWTPs) are assumed to be one of the main sources of various micropollutants in aquatic environments through their insufficient cleaning processes [1-5]. Most WWTPs are not designed to completely eliminate micropollutants, especially when only conventional processes are employed. The removal efficiency of WWTPs varies depending on the physicochemical characteristics of the pollutants and on the treatment processes involved [1,2]. Secondary treatment (mostly in activated sludge or membrane biological reactors) is the main mechanism of pollutant removal in conventional WWTPs [6,7]. Application of additional treatments, also called tertiary treatment or advanced treatment, in WWTP processes might improve pollutant removal [1,2]. Advanced treatments include natural systems (e.g. constructed wetlands, aquifer recharge and recovery [8]), membrane and advanced chemical/oxidation technologies [9], electro-oxidation [10] and biological treatment [11-13]. The cost of most processes listed above limits their broad full-scale application.

Endocrine disrupting compounds (EDCs) belong among the most recently targeted micropollutants detected in WWTP effluents and also in aquatic environments. EDCs are specific in

their high biological activities towards various organisms. The most adverse and risky effect lies in their ability to cause reproductive problems in a number of species and probably also in humans. The estrogenic effect of EDCs is often expressed in terms of the estradiol equivalent (EEQ) and it has been documented that a concentration of 1 ng L^{-1} EEQ has a significant negative effect on fish and other aquatic organisms [14,15]. Due to their high estrogenic activity, estrone (E1), 17β-estradiol (E2), estriol (E3) and synthetic 17α-ethinylestradiol (EE2) are considered to be significant contributors to the estrogenic activity of wastewaters. Bisphenol A (BPA) and 4-n-nonylphenol (4-NP) have many orders of magnitude lower estrogenic activity, but their elevated concentrations in wastewater also draw attention to these EDCs [16].

In this context, the search for environmentally friendly and low-cost technologies is of great importance. Bioremediation is a popular alternative to conventional treatment methods and especially white rot fungi (WRF) have been successfully documented for their ability to remove various organic pollutants i.e. PAHs, PCBs, textile dyes, pesticides, as well as EDCs (as described in a review elsewhere [17-20]).

The white rot fungus *Pleurotus* sp., also called oyster, abalone or tree mushroom, is one of the most commonly cultivated edible mushrooms in the world. Mushroom farming is a profitable business and because *Pleurotus* (mainly *Pleurotus ostreatus*) is fairly easy to cultivate and to fructificate, there are many growing farms around the world (mainly in Europe and the U.S.A.), producing fruiting bodies as well as tons of re-usable biowaste. This biowaste usually consists of ligno-cellulosic substrate (straw, wood chips, fruit waste etc.) and *Pleurotus* mycelia. Because of the great bioremediation potential of *Pleurotus ostreatus* [20-22], re-use of this biological waste in organic pollutant degradation represents a promising environmentally friendly technology.

This study was performed to examine the degradation of the main representatives of endocrine disrupting compounds by the commercially available, edible white rot *P. ostreatus* strain HK 35 in augmented bioreactors. The experiments were designed to explore the degradation ability under various regimes and conditions, including different matrices (tap water and wastewater) representing distinct microbial populations as well as different EDC concentration levels in fortified and real wastewater. Initially the strain was compared with the known 3004 strain described in the literature for its degradation efficiency under model laboratory conditions. In the next step, the strain was tested in a laboratory-scale continuous-flow reactor. Then the strain was examined in a scaled-up reactor on a stationary packed bed and for continuous flow trickle-bed regimes. Finally, the function of the trickle-bed arrangement was verified at a WWTP locality. The removal process and its efficiency were assessed using various methods including analytical EDC determination, detection of residual estrogenic activity, ligninolytic enzyme activity and phospholipid fatty acid (PLFA) analysis, in order to monitor the fungal and bacterial biomass.

Materials and Methods

Materials and substrate preparation

The spent straw substrate containing the fungal biomass that was utilized in the experiments was obtained from a commercial oyster mushroom farm (Farma Volek, CZ). Wheat straw pellets (8 mm Ø), used as a bulking agent and a fresh nutrition source, were purchased from Atea Praha (CZ). The straw pellets were moisturized with distilled water (1:3 *w/w*), twice sterilized in an autoclave (121°C; 25 min) and stored at room temperature (max 24 hours). For the degradation experiments, the spent straw substrate and sterile straw pellets were mixed in a ratio of 2:1 (*w/w*) and left for 3 days at 28°C in the darkness. In order to remove color pigments originating from straw, the fungal straw substrate was washed with tap water (1:2; *w/w*), before the experiments. This material was used as the filling for all the reactor experiments.

Wastewater was obtained from a WWTP located in Central Bohemia. WWTP effluent after secondary treatment, which is normally discharged into a recipient, was stored in a stainless tank at 4°C and used as a matrix in laboratory experiments.

Bisphenol A (BPA); estrone (E1), 17β-estradiol (E2), estriol (E3), 17α-ethinylestradiol (EE2), triclosan (TRC) and 4-n-nonylphenol (4-NP) were used as analytical standards and as substrates for the degradation experiments. All the compounds were purchased from Sigma Aldrich (Steinheim, Germany) with purity of 99.0% or higher. Standard solutions containing 2 mg mL^{-1} of each of the six selected EDCs were prepared in ethyl acetate (EtOAc) and stored in a refrigerator. The stock standard solutions with the respective concentrations were prepared by mixing all 7 EDCs and diluting the stock solution with EtOAc or dimethylsulfoxide (DMSO) or N,N-dimethylformamide (DMF).

All the analytical grade organic solvents used in present study were purchased from Chromservis (CZ). The analytical standards of fatty acid methyl esters were obtained from Sigma-Aldrich (Prague, Czech Republic) and Matreya LLC (USA). BSTFA:TMCS (99:1); glucose, agar, malt extract broth, DMSO, N,N-dimethylformamide and all the other chemical used in the present study were purchased from Sigma (Germany).

Fungal culture

The spent straw substrate obtained from the mushroom farm was colonized by industrial *P. ostreatus* HK 35 and stored at 4°C. The strain was aseptically isolated on Petri dishes with MEG agar medium (malt extract 20 g L^{-1}, glucose 20 g L^{-1}, agar 25 g L^{-1}, pH adjusted to 6.0 with NaOH prior to autoclaving) and stored at 4°C. After one week, the strain was reinoculated on Petri dishes with fresh MEG medium; the colonized Petri dishes were transferred to 4°C and used as stock culture. Every two months, the fungus was re-inoculated on fresh medium.

P. ostreatus 3004 was obtained from the Culture Collection of Basidiomycetes of the Czech Academy of Sciences (*P. ostreatus* 3004 CCBAS 278).

The fungal inocula for biodegradation experiments were prepared in 250 mL Erlenmeyer flasks containing 20 mL of malt extract-glucose medium (MEG; 5 g malt extract, 10 g glucose, pH 4.5) starting from 2 mycelial plugs (7 mm Ø). The cultures were grown for 7 days at 28°C, homogenized by Ultraturrax-T25 (IKA-Labortechnik, Germany) and 5% aliquots of the mycelial suspension were used to inoculate sterile MEG medium.

In vivo biodegradation under sterile conditions

The static MEG medium liquid cultures of *P. ostreatus* 3004 and HK 35 were incubated in 250 mL Erlenmeyer flasks at 28°C in five parallel samples. The samples for biodegradation experiments were spiked with 100 µL of DMF (final concentration 0.5%, v/v; Sigma Germany) containing a mixture of EDCs (final concentration 2 mg L^{-1} of each representative). Biotic controls were spiked with only 100 µL of DMF. The abiotic controls were performed with mycelia grown for one week and killed in an autoclave. The biodegradation samples were harvested after 3, 12 and 20 d, extracted according to [23] and analyzed using the GC/MS method (see below). All the samples were prepared in triplicate.

Laboratory-scale continuous flow trickle bed bioreactor

A laboratory-scale glass tubular, vertical reactor with total volume of 1 L (working volume 0.8 L) was filled with the mixture of straw substrates (total amount 200 g of wet substrate, 75% humidity), washed with tap water (2 L) and aerated using a small air pump (3 L min^{-1}; Hailea Aco-6601, Hailea, CZ). These experiments were carried out with fortified WWTP effluent (original EDC concentration sum 356 ng L^{-1}; fortification level 500 µg of individual EDCs; total volume of wastewater 400 mL) under non-sterile conditions. The continuous flow was set at 0.3 L h^{-1} and was maintained for 2 days using a peristaltic pump (Watson Marlow, 114VD; AxFlow, CZ). The effluent was collected in a glass reservoir and pumped again through the bioreactor in a cycle. In order to assess the degradation efficiency, the effluent was repeatedly sampled (4 mL) throughout the experiment (2 days). The experiment was performed in 5 parallel measurements. The samples were directly extracted using liquid-liquid extraction (LLE) with EtOAc, derivatized and analyzed using GC/MS method (see below).

Pilot-scale static packed bed bioreactor – batch mode with tap-water and wastewater

A pilot-scale polycarbonate cylindrical vertical reactor with a total volume of 47 L (working volume 30 L) was filled with the mixture of straw substrates (total amount 12 kg of wet substrate, 75% humidity), equipped with an air pump (Watson Marlow, 114VD; AxFlow, CZ) and diffuser placed in the bottom of the reactor (100 L min^{-1}) and placed in an iron frame holder in the laboratory. Two individual experiments with different microbial matrices were carried out separately in the batch arrangement using tap water and wastewater. The fortification level was 60 µg L^{-1} of each EDC. The total volume of each the water sample was 6 L. The bioreactor operated for 10 days and samples were collected from the bottom of the reactor during this period.

Pilot-scale trickle-bed bioreactor with WWTP effluent and fortified WWTP effluent

The same pilot-scale polycarbonate cylindrical reactor was equipped with a PTFE dispenser with 20 stainless needless, which was placed at the top of the reactor to uniformly distribute the liquid over the whole substrate surface in the trickle-bed reactor arrangement.

The experiments were carried out with real wastewater (EDC sum 455 ng L^{-1}) and also with fortified wastewater (200 µg L^{-1} of each EDC), both under non-sterile conditions (total volume 6 L). The flow rate setting was 2 L h^{-1} using a peristaltic pump (Watson Marlow 120, AxFlow, CZ). The effluent was collected in a glass reservoir and was repeatedly sampled for the analyses. The hydraulic retention time was approx. 5 minutes. Samples were collected from a glass reservoir, in which the bioreactor effluent was pooled and stored.

Pilot-scale trickle bed bioreactor on WWTP locality

The pilot-scale polycarbonate cylindrical reactor was placed in the vicinity of a WWTP in Central Bohemia and the reactor was connected to the WWTP effluent, which is commonly discharged into the local recipient. The bioreactor was installed close to the WWTP together with a mobile container fully equipped with technical facilities for operating the reactor. The bioreactor effluent was pooled in a stainless steel reservoir (100 L volume). The reactor was prepared according to PS-TBR in the laboratory. The real wastewater influent and effluent were sampled every 60 minutes and the collected samples were pooled together to create representative 48-hour samples; the reactor efficiency was monitored for 14 days. Each sample was analyzed in four replicates. The reactor influent flow rate varied from 0.64 L h^{-1} up to 3.60 L h^{-1}.

Microbial biomass quantification - PLFA analysis

Phospholipid fatty acid analysis (PLFA) was employed to estimate changes in the microbial community of the fungal bioreactor operating under non-sterile conditions. The straw samples were lyophilized (Labio, ČR) and homogenized before PLFA extraction.

PLFA was performed using LLE extraction with a chloroform–methanol–phosphate buffer mixture (1:2:0.8 *v/v*) and the extracted lipids were purified using solid-phase extraction cartridges (LiChrolut Si 60, Merck, Germany) according to [24]. Samples then underwent mild alkaline methanolysis and the methylated PLFAs were analyzed by GC/MS. The fungal biomass was quantified based on the 18:2ω6,9 content; the bacterial biomass was quantified as the sum of i14:0, i15:0, a15:0, 16:1ω5, 16:1ω7, 16:1ω9, 10Me-16:0, i16:0, i17:0, a17:0, cy17:0, 17:0, 10Me-17:0, 18:1ω7, 10Me-18:0, cy19:0 (Gram+ i14:0, i15:0, a15:0, i16:0, i17:0, a17:0; Gram− 16:1ω7, 16:1ω9, 18:1ω7, cy17:0, cy19:0) as reported elsewhere [25].

EDC extraction and analysis

EDC extraction from the water samples was carried out using SPE columns (C18 Chromabond; Chromservis, CZ). After conditioning the SPE columns (EtOAc, methanol, water), 1000 mL of filtered (0.22 µm; Whatman nylon membrane filters, UK) acidified wastewater (pH 2.5, adjusted with HCl) was applied. The samples were eluted with 10 mL of EtOAc, dried with sodium sulfate, purified using gel permeation chromatography (GPC gel BioBeads S-X12; Chromservis, CZ), trimethylasillylated (using BSTFA; 60°C; 30 min) and analyzed by GC/MS (SCION SQ Bruker; USA).

EDC extraction from the straw substrate was carried out using the ASE 200 accelerated solvent extractor (Dionex, France). ASE samples were prepared by weighing 1 g of previously lyophilized (Labio, ČR) and homogenized straw material placed in the ASE cell (11 mL volume). The extraction was performed at temperature of 150°C and pressure of 10.34 MPa with EtOAc using 3 cycles. The final extracts were concentrated using a nitrogen stream.

Separation of the EDC mixture was performed using the Rxi-5ms column (30 m x 0.25 µm, 0.25 mm ID; Restek, USA). Helium 5.0 (99.999% purity; Linde, CZ) was used as the carrier gas with a constant flow rate of 1 mL min^{-1}. The injector was operated in the split/splitless mode, with splitless time of 1 min. The injector temperature was 240°C, the EI source, transferline and manifold temperatures were 250, 280 and 50°C, respectively. The GC oven temperature program started from 60°C (for 1 min), then heated up to 120°C at 25°C min^{-1}, and finally to 240°C at 2.5°C min^{-1}, where it was held isothermally for 28 min. The mass spectra were recorded at 3 scans min^{-1} under electron impact of 70 eV. For qualitative analysis, the full scan mode (50-750 amu) was used. The selected ion monitoring (SIM) mode, based on the most abundant ions, was used for quantitative analysis. The selected ions were: 179 and 292 for 4-NP; 357 for BPA; 368 for internal standard BPA-d_{16}; 342+257+244 for E1; 416+26+285 for E2; 504+415+346 for E3; 196+425 for EE2; 345:349 for TRC; 282:288 for internal standard hexachlorobenzene (HCB; Sigma, Germany).

Enzyme activity and estrogenic activity determination

The laccase activity was determined by oxidation of 5 mM 2,2-azinobis-3-ethylbenzo-thiazoline-6-sulfonic acid [26]. One unit of enzyme produced 1 µmol of the reaction product per min under the assay conditions at room temperature.

The estrogen-like activity of the tested samples was measured using standardized yeast estrogenic (YES) assay with the recombinant strain of *Saccharomyces cerevisiae*, producing β-galactosidase in response to estrogen exposure [27]. Prior to the analysis, the sample extracts in EtOAc were evaporated and re-dissolved in 30% DMSO. Serial dilutions of the samples were also performed using 30% DMSO.

The test was performed according to [28]. Serial dilutions of natural estrogen estradiol were measured at each plate along with the samples. 7 concentrations with 2-fold dilution were measured to obtain the whole dose-response curve of estradiol. 3% DMSO served as a blank. All the measurements were performed in triplicate. Various dilutions were plotted against the correlated absorbance to obtain the dose-response curve for each sample. The EEQ values were calculated using dose-response curves of samples and E2 [27]. The EEQ value indicates that the sample has the same estrogenic activity as a stock solution containing this concentration of E2.

Results and discussion

Comparison of P. ostreatus HK 35 with 3004 strains under model laboratory conditions

The degradation potential of *P. ostreatus* HK 35 was assessed using the laboratory *in vivo* test. *P. ostreatus* HK 35 is an edible industrial strain cultivated for alimentary purposes in commercial oyster mushroom growing farms. Its degradation potential was compared to *P. ostreatus* 3004. The model laboratory degradation experiment showed that both the tested strains were able to degrade all the tested EDCs (Figure 1). *P. ostreatus* HK 35, obtained from a growing farm exhibited even higher degradation ability for the EDC mixture than the 3004 strain. It is worth noting that the degradation potential of *P. ostreatus* 3004 towards various organopollutants including EDCs have been documented in various articles published by our group [21,23,29-31]. Generally, high degradation efficiency of *P. ostreatus* was also reported by many other authors [32-35]. As previously reported, EDCs are able to influence the enzyme activity profiles [23] of WRF. In the current study, no significant decrease in the laccase activity was observed in both the *P. ostreatus* cultures (3004 and HK 35) in the presence of EDC (data not showed). Based on the results of this experiment, the HK 35 strain in the form of the spent substrate was used for the other bioreactor experiments.

Degradation of EDCs in a laboratory-scale continuous-flow trickle-bed bioreactor

This experiment was performed to explore whether the HK 35 strain is able to decompose EDCs under non-sterile conditions on a small laboratory scale. Spent fungal substrate from a commercial oyster mushroom-growing farm mixed with wheat straw pellets (see section 2.1) was used as a reactor carrier material and also as nutrient and culture sources. The substrate was highly colonized by *P. ostreatus* HK 35 mycelium. Real WWTP effluent was used without any treatment, thus autochthonous microorganisms as well as suspended solid matter, organic and inorganic compounds derived from WWTP process were present.

The non-sterile batch reactor comprising a mixed culture of the white rot fungus and bacteria showed a large decrease in the EDC concentration in the effluent within the first 3 hours (to below 41% of the initial concentration). Although the initial degradation rate varied among the individual reactors (Fig. 2), the residual concentration after 18 h dropped below 10% in all cases. The variability in the degradation rate can by caused by slight inhomogeneity in the fungal colonization during the initial stage. Fortified WWTP effluent (total volume 400 mL) was circulating in the system for 48 hours. The lacccase activity was detected in all the reactor effluents at a level of units per liter.

EDC degradation in a pilot-scale static packed-bed bioreactor – batch mode with tap water and wastewater

These experiments were performed in order to assess whether scaling up the process could negatively effect the degradation efficiency of the fungus. Attention was also paid to the influence of the autochthonous microflora present. For this purpose, two different strategies for the batch mode evaluation were employed using the tap-water and wastewater matrices. High degradation efficiency was observed for both the experiments when more than 78% of initial EDCs were removed within the first 2 hours in the fortified tap water and more than 91% were removed in the fortified wastewater. After 24 hours, almost total degradation (more than 97%) of EDCs was determined.

The results of the PLFA analysis showed that the fungus was able to thrive in the presence of wastewater bacteria. The data revealed that the fungus was able not only to survive but also to be biologically active under these conditions, which is documented by very similar degradation efficiencies under both the conditions (Fig. 2 and 3). Moreover, the results showed that the fungus is also very efficient in suppressing estrogenic activity, as is documented in Fig. 3 and 5. The pattern of the EEQ removal followed the EDC degradation [23].

Interspecific interactions of WRF and bacterial strains have already been documented in several publications (reviewed in [36]). Borras et al. [37] documented a negative effect on laccase production by *Trametes versicolor* in the presence of two soil bacteria. Since laccase participates in the degradation of many organic pollutants [38,39], the interaction of white-rot fungi and other microorganisms can also affect the biodegradation of organic pollutants. On the other hand, in another work, Baldrian et al. [40] studied the effect of interspecific interactions between WRF and other microorganisms on laccase activity and concluded that the induction of laccase activity is a typical response of a wide range of WRF to interspecific interactions. In the present study, a significant increase (ANOVA, $p<0.05$) in laccase activity was observed after 2 days of wastewater treatment in the bioreactor (124 U mg^{-1} of 18:2ω6,9) compared to the sterile reactor (56 U mg^{-1} of 18:2ω6,9). No significant difference in laccase activity was observed on other sampling days and the laccase activity was about 60 U mg^{-1} of 18:2ω6,9 for both reactors.

P. ostreatus biomass estimated as PLFA concentrations had a slightly increasing tendency at the beginning of both the experiments; however, at the end the fungal biomass similarly reached approx. 100 μg g^{-1} of the straw substrate (Fig. 4). In contrast, the bacterial biomass exhibited an increasing trend during the whole duration of the tests. It is worth noting that, as expected, the bacterial concentrations in the wastewater matrix were almost twice as high as those in the tap water at the end of the experiment. The results further showed that the fungal/bacterial ratio decreased during the experiment and especially Gram negative bacteria were responsible for the bacterial biomass increase (data not shown). A certain tolerance of *P. ostreatus* toward soil bacteria, in contrast to another highly degrading white species (*Irpex lacteus*), was recorded in a work by Stella et al. (in press). Despite the fact that bacterial degradation is probably less effective, as was demonstrated in

previously published papers [2,41,42] and fungal biodegradation is probably the major EDC elimination mechanism in this study, the PLFA results indicate that the fungus was not substantially negatively influenced by the autochthonous bacterial microflora.

Pilot-scale trickle-bed bioreactor with WWTP effluent and fortified WWTP effluent

The set of these experiments was intended to test the EDC degradation ability of the fungal strain exposed to real wastewater on a pilot scale in the trickle-bed arrangement. In this case, the results also revealed that the EDC degradation in the trickle-bed bioreactor containing the mixed culture of the fungus and wastewater autochthonous bacteria was very efficient. The results representing the fortified wastewater are shown in Fig. 6. It is noteworthy that the whole volume of the water sample passed through the bioreactor within 1 hour and the effluent was collected in the reservoir. The subsequent degradation continued via secreted ligninolytic enzymes, where mainly laccase activity ranged from 10^1 to 10^2 U L^{-1}. As in the previous experiment, the residual estrogenic activity followed the pattern of the EDC removal; however, the percentage values were higher than expected according to the analytical results. This discrepancy indicates the temporary formation of metabolites with the estrogenic activity that has already been described. In our previous study [21] several transformation products of EE2 were identified and described. The metabolites originated from *in vivo* and *in vitro* degradation of EE2 using the *P. ostreatus* 3004 fungal strain.

In order to document that biological degradation was the main mechanism involved, control analyses of the straw substrate were performed using accelerated solvent extraction. Straw samples (approx. 4 g) were repeatedly collected through installed ports during the whole bioreactor operating period from the bottom, top and the middle of the reactor, lyophilized, homogenized, extracted using ASE and analyzed using GC/MS. Since no EDCs were detected, sorption was excluded as the removal mechanism.

An experiment with the same trickle-bed reactor arrangement was performed with an unfortified WWTP effluent representing real environmental concentrations. The initial concentrations of EDCs in the WWTP effluent are listed in Table 1 – Time 0h. Except E1, no natural estrogens used in this study were observed. Substantial removal (more than 70% of the initial concentration) was observed after 48-hour treatment for the synthetic contraceptive EE2 and other tested compounds (BPA, 4-NP and TRC). Because of the very low concentration of EDCs present in the reactor influent, no estrogenic activity was detected using the recombinant yeast assay either in the reactor influent or in the reactor effluent (data not shown).

Pilot-scale trickle-bed bioreactor in the WWTP vicinity - tertiary treatment

A pilot-scale reactor in the trickle-bed arrangement was installed in the vicinity of a WWTP. The bioreactor was employed as a tertiary treatment step to remove EDCs present in the wastewater. The effluent from the WWTP (after the secondary treatment) and effluents from the reactor (after tertiary treatment) were systematically analyzed.

Natural estrogens E2 and E3 were not present in the WWTP effluent of any sampling campaign. Other tested EDCs were detected in concentrations from 7.5 to 100.6 ng L^{-1} after the secondary treatment. After the tertiary treatment, the EDC concentrations ranged from below their respective limits of detection to 22.6 ng L^{-1}. The most abundant compound was BPA in both cases. Due to the very low concentration of mammalian and synthetic hormones, which are the major contributors to the total estrogenicity of wastewaters [43,44], the estrogenic activities were below or close to the detection limit of the YES test (data not shown). *P. ostreatus* HK 35 removed 76% of the influent EDC concentration within 24 hours. The results displayed in Fig.6 represent the data for the 10-day experiment when the collected 24 hour samples were analyzed as a single composite sample in four replicates. The lowest degradation rate was observed for TRC (15%), but the degradation varied greatly (7% to 46%). The activity of laccase in the sample collection reservoir was stable during the individual 24-hour cycles and ranged from 115 to 135 U L^{-1}.

Conclusions

The results of this study document that the widely available strain *P. ostreatus* HK 35 is efficient in biodegradation of typical EDC representatives including their endocrine-active metabolites. The data revealed that the fungus is able to operate under various static and continuous-flow regimes of the bioreactors and the degradation efficiency is not negatively influenced by bacterial microflora present in the wastewater. Moreover, the set of experiments demonstrated the feasibility of scaling up the process. The final tested parameters of the bioreactor at the WWTP locality are still far from practical application in communal wastewater treatment; however, its application can be easily focused on smaller WWTPs used in industry or hospitals. The main advantage of the strain is its general availability and the fact that the spent substrate represents waste that can be easily utilized for wastewater treatment processes. The results of this study emphasize the need for further research to optimize the use of this spent substrate. Thus it is necessary to investigate further the applicable duration of the treatment process and possibly factors (e.g. additional lignocellulose substrates) that can favorably influence this parameter. Another direction of research could be investigation of other organic pollutants in wastewater and the ability of *P. ostreatus* to decompose them. Although the biodegradation properties of this fungus have been documented in the literature, most studies were performed under model conditions and only documented the types and categories of pollutants that can be treated with the fungus. On the other hand, the influence of real environmental conditions must be tested in order to ensure the applicability of the fungus.

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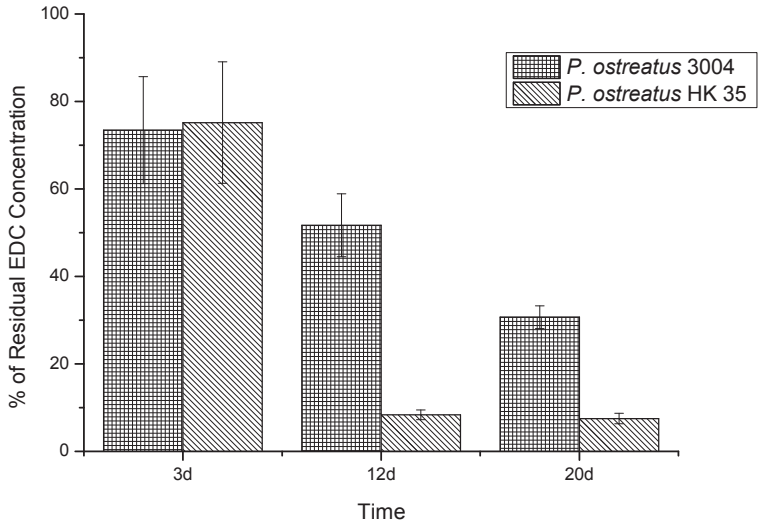

Fig. 1 *In vivo* degradation of the EDC mixture using two different *P. ostreatus* strains. EDCs concentration is expressed as the sum of 7 individual representatives in the respective cultures 3 days, 12 days and 20 days after inoculation. The error bars represent the standard deviations of three independent cultivation samples.

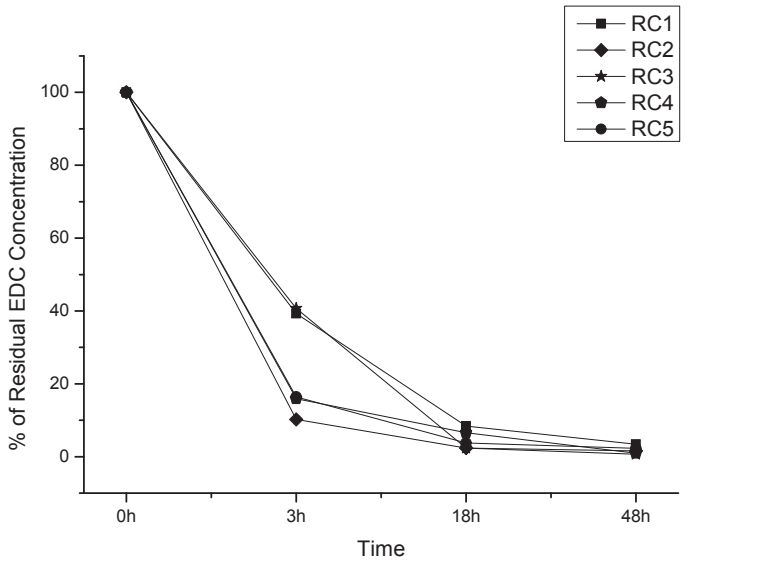

Fig. 2 EDC degradation in a laboratory-scale continuous flow trickle bed bioreactor. The data represent the results of 5 individual reactor runs (RC1-RC5). The concentration of the sum of EDCs is expressed as a percentage of the initial conditions (time 0). The initial amount was 200 µg of each EDC representative spiked into the real WWTP effluent (400 mL). The flow rate was 0.3 L h^{-1}. Each point represents the mean of 3 independent analyses (the relative standard deviations were below 24%).

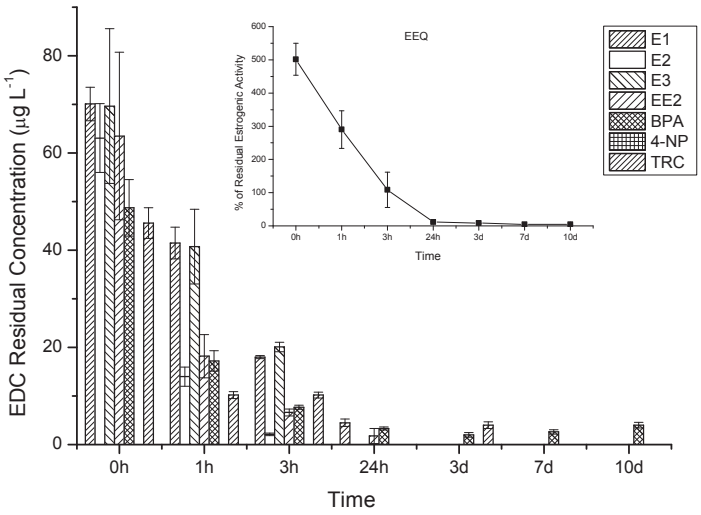

Fig. 3 EDC degradation in the pilot-scale static packed-bed bioreactor in the batch mode with the fortified tap water. Fortification level 60 μg L^{-1} of each EDC. The degradation is expressed as a percentage of the EDC influent concentrations. The curve of EEQ (estrogenic equivalents) shows a decrease in the estrogenic activity during the reactor run. The data represent the means of 3 independent analyses and the error bar represents the standard deviation.

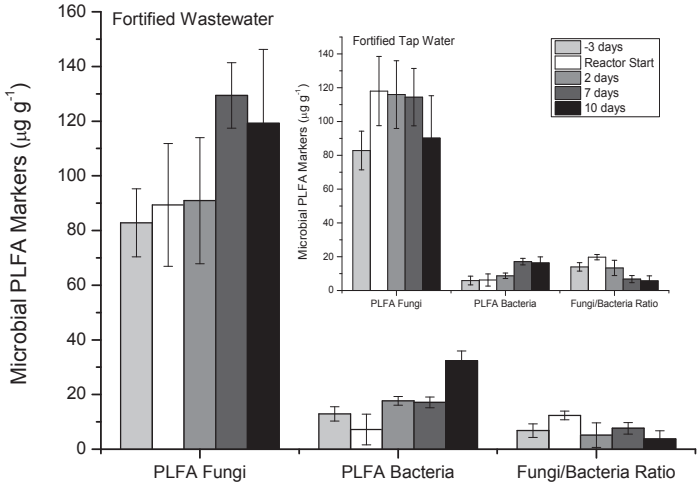

Fig. 4 The results of the PLFA analysis in the fortified tap-water (A) and fortified wastewater (B) detected in the pilot-scale static packed bed bioreactor. The error bar represents the standard deviation (n=3).

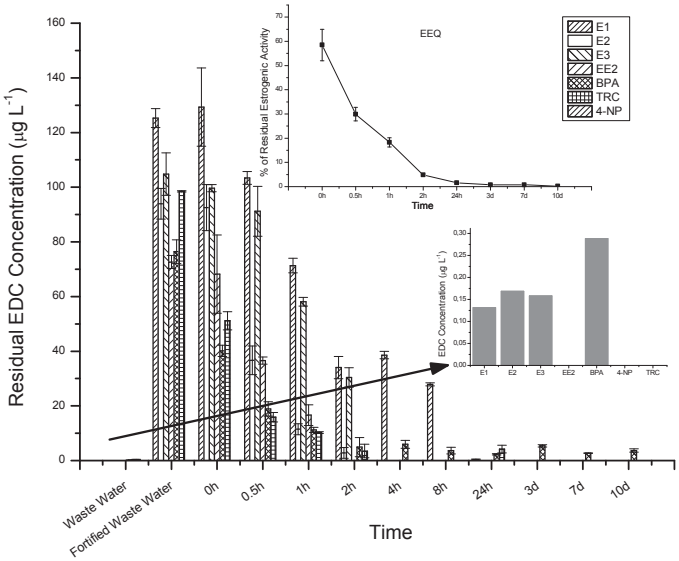

Fig. 5 EDC degradation in the pilot-scale static packed-bed bioreactor in the batch mode with the fortified wastewater. The degradation is expressed as a percentage of the EDC influent concentrations. The curve of EEQ (estrogenic equivalents) shows a decrease in estrogenic activity during the reactor run. The data represent the means of 3 independent analyses and the error bar represents the standard deviation. Concentrations of the original EDC contamination are displayed on the right as the grey column chart.

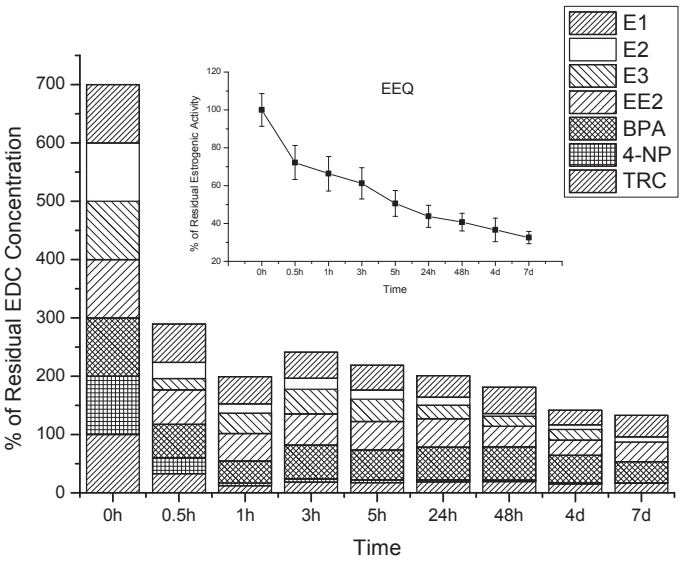

Fig. 6 EDC biodegradation and estrogenic activity (expressed as estrogenic equivalents - EEQ) removal in the pilot-scale trickle-bed bioreactor in the fortified wastewater. The degradation and the EEQ decrease are expressed as a percentage of the EDC sum and the initial EEQ values in the reactor influent. The bioreactor influent was formed from real WWTP effluent and was fortified to 200 µg L^{-1}; flow rate 2 L h^{-1}. The error bars represent the relative standard deviations for three individual bioreactor cycles.

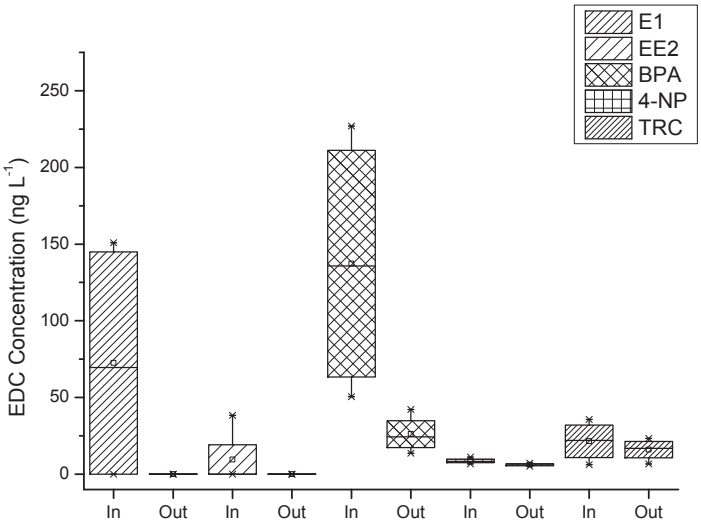

Fig. 7 Box plots of the individual EDC concentrations in the reactor influent (In) and the effluent (Out) in the 10-day experiment. Each box displays the median, upper and lower quartiles of the respective dataset. Box whiskers represent the maximum and minimum range excluding any outlier (shown as a dagger). E2 and E3 were not detected either in the influent or in the effluent. (n=10)

Table 1 EDC degradation in real wastewater using a pilot-scale reactor in the trickle-bed arrangement. The data represent the means of 3 parallel bioreactors using the same WWTP effluent (Time 0 h).

		EDC degradation in WWTP effluent using a pilot-scale bioreactor							
		E1	E2	E3	EE2	BPA	4-NP	TRC	Sum of EDCs
	Time	ng L^{-1}	ng L^{-1}	ng L^{-1}	ng L^{-1}	ng L^{-1}	ng L^{-1}	ng L^{-1}	ng L^{-1}
Wastewater	0 h	60.7	ND	ND	16.0	80.6	9.6	20.9	187.8
	24 h	ND	ND	ND	7.1±0.5	25.4±2.9	3.5±0.6	7.3±1.7	43.2±5.7
	48 h	ND	ND	ND	4.5±1.0	18.6±2.9	ND	5.0±1.8	28.1±5.7

ND – not detected