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RESEARCH ARTICLE

Toxicity, dioxin-like activities, and endocrine effects of DDT metabolites—DDA, DDMU, DDMS, and DDCN

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Abstract
Background, aim, and scope 2,2-*bis*(chlorophenyl)-1,1,1-trichloroethane (DDT) metabolites, other than those routinely measured [i.e., 2,2-*bis*(chlorophenyl)-1,1-dichloroethylene (DDE) and 2,2-*bis*(chlorophenyl)-1,1-dichloroethane (DDD)], have recently been detected in elevated concentrations not only in the surface water of Teltow Canal, Berlin, but also in sediment samples from Elbe tributaries (e.g., Mulde and Havel/Spree). This was paralleled by recent reports that multiple other metabolites could emerge from the degradation of parent DDT by naturally occurring organisms or by interaction with some heavy metals. Nevertheless, only very few data on the biological activities of these metabolites are available to date. The objective of this communication is to evaluate, for the first time, the cytotoxicity, dioxin-like activity, and estrogenicity of the least-studied DDT metabolites.
Methods Four DDT metabolites, *p,p′*-2,2-*bis*(chlorophenyl)-1-chloroethylene (DDMU), *p,p′*-2,2-*bis*(chlorophenyl)-1-chloroethane (DDMS), *p,p′*-2,2-*bis*(4-ch1oropheny1)acetonitrile (DDCN), and *p,p′*-2,2-*bis*(chlorophenyl)acetic acid (DDA), were selected based on their presence in environmental samples in Germany such as in sediments from the Mulde River and Teltow Canal. *O,p′*-DDT was used as reference in all assays. Cytotoxicity was measured by neutral red retention with the permanent cell line RTG-2 of rainbow

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trout (*Oncorhynchus mykiss*). Dioxin-like activity was determined using the 7-ethoxyresorufin-*O*-deetylase assay. The estrogenic potential was tested in a dot blot/RNAse protection-assay with primary hepatocytes from male rainbow trout (*O. mykiss*) and in a yeast estrogen screen (YES) assay.

Results All DDT metabolites tested revealed a clear dose–response relationship for cytotoxicity in RTG-2 cells, but no dioxin-like activities with RTL-W1 cells. The dot blot/RNAse protection-assay demonstrated that the highest non-toxic concentrations of these DDT metabolites (50 μM) had vitellogenin-induction potentials comparable to the positive control (1 nM 17β-estradiol). The estrogenic activities could be ranked as *o,p′*-DDT > *p,p′*-DDMS > *p,p′*-DDMU > *p,p′*-DDCN. In contrast, *p,p′*-DDA showed a moderate anti-estrogenic effect. In the YES assay, besides the reference *o,p′*-DDT, *p,p′*-DDMS and *p,p′*-DDMU displayed dose-dependent estrogenic potentials, whereas *p,p′*-DDCN and *p,p′*-DDA did not show any estrogenic potential.

Discussion The reference toxicant *o,p′*-DDT displayed a similar spectrum of estrogenic activities similar to 17β-estradiol, however, with a lower potency. Both *p,p′*-DDMS and *p,p′*-DDMU were also shown to have dose-dependent estrogenic potentials, which were much lower than the reference *o,p′*-DDT, in both the vitellogenin and YES bioassays. Interestingly, *p,p′*-DDA did not show estrogenic activity but rather displayed a tendency towards anti-estrogenic activity by inhibiting the estrogenic effect of 17β-estradiol. The results also showed that the *p,p′*-metabolites DDMU, DDMS, DDCN, and DDA do not show any dioxin-like activities in RTL-W1 cells, thus resembling the major DDT metabolites DDD and DDE.

Conclusions All the DDT metabolites tested did not exhibit dioxin-like activities in RTL-W1 cells, but show cytotoxic and estrogenic activities. Based on the results of the in vitro assays used in our study and on the reported concentrations of DDT metabolites in contaminated sediments, such substances could, in the future, pose interference with the normal reproductive and endocrine functions in various organisms exposed to these chemicals. Consequently, there is an urgent need to examine more comprehensively the risk of environmental concentrations of the investigated DDT metabolites using in vivo studies. However, this should be paralleled also by periodic evaluation and monitoring of the current levels of the DDT metabolites in environmental matrices.

Recommendations and perspectives Our results clearly point out the need to integrate the potential ecotoxicological risks associated with the "neglected" *p,p′*-DDT metabolites. For instance, these DDT metabolites should be integrated into sediment risk assessment initiatives in contaminated areas. One major challenge would be the identification of baseline data for such risk assessment. Further studies are also warranted to determine possible additive, synergistic, or antagonistic effects that may interfere with the fundamental cytotoxicity and endocrine activities of these metabolites. For a more conclusive assessment of the spectrum of DDT metabolites, additional bioassays are needed to identify potential anti-estrogenic, androgenic, and/or anti-androgenic effects.

Keywords Cytotoxicity · DDA · DDCN · DDMS · DDT metabolites · DDMU · Dioxin-like activity · Dot blot/RNAse protection-assay · EROD · Estrogenic activity · Yeast estrogen screen

Abbreviations

BH	Benzhydrole
BP	Benzophenone
DBH	2,2-*bis*(chlorophenyl)benzhydrole
DBP	2,2-*bis*(chlorophenyl)benzophenone
DDA	2,2-*bis*(chlorophenyl)acetic acid
DDCN	2,2-*bis*(4-chlorophenyl)acetonitrile
DDD	2,2-*bis*(chlorophenyl)-1,1-dichloroethane
DDE	2,2-*bis*(chlorophenyl)-1,1-dichloroethylene
DDEt	2,2-*bis*(chlorophenyl)ethane
DDM	2,2-*bis*(chlorophenyl)methane
DDMS	2,2-*bis*(chlorophenyl)-1-chloroethane
DDMU	2,2-*bis*(chlorophenyl)-1-chloroethylene
DDNU	2,2-*bis*(chlorophenyl)ethene
DDOH	2,2-*bis*(chlorophenyl)-1-ethanol
DDPS	1,1-*bis*(4-chlorophenyl)-1-propene)
DDPU	3,3-*bis*(4-chlorophenyl)-1-propene)
DDT	2,2-*bis*(chlorophenyl)-1,1,1-trichloroethane
DDX	DDT-related compound
DMSO	Dimethylsulfoxide
FRG	Federal Republic of Germany
NOEC	No observed effect concentration

1 Introduction

Since its introduction as an insecticide in 1939, the chlororganic compound 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) has been extensively used worldwide for controlling agricultural pests and arthropod disease-causing vectors (Metcalf 1973). However, problems associated with continuous use and applications of DDT and its metabolites came up in the early 1960s (Kale et al. 1999), when DDT was found to induce environmental damages while at the same time, posing a threat to the health of various organisms (e.g., Fry and Toone 1981; Sharpe 1995; Megharaj et al. 2000). The harmful properties of DDT and its metabolites are due to their highly lipophilic nature (e.g., Lotufo et al. 2001) facilitating their accumulation, persis-

tence, and stability (Voldner and Li 1995), as well as their biomagnification along the food chain (Falandysz et al. 2001). Toxicological effects on individual organisms as well as complex ecosystems have been widely documented, e.g., endocrine effects, reproductive toxicity (Vos et al. 2000), and, most recently, genotoxicity (Binelli et al. 2008). After the ban of DDT and its metabolites in the USA in 1972, and subsequently in many other countries, the US Environmental Protection Agency has declared DDT as a priority pollutant (Keith and Telliard 1979). More recently, the Stockholm Convention in 2002 listed DDT among the so-called Dirty Dozen. Nonetheless, the unabated use of DDT for public health purposes, especially in many malaria-affected countries, poses a significant continuing environmental challenge (Lotufo et al. 2000).

DDT and its metabolites have been recognized as widely distributed and highly stable environmental contaminants, and their persistence especially in cold climates, are of worldwide concern. They still remain in aquatic sediments more than 20 years after the use of DDT was banned in the USA (Eganhouse and Pontolillo 2000, 2008), Europe (Dünnbier et al. 1997), and other countries (e.g., Phuong et al. 1998). Even in the Arctic ecosystem, DDT has been consistently detected at elevated concentrations (e.g., Dietz et al. 2000). DDT's wide distribution in the environment is primarily due to its application as an agricultural pesticide. Peak concentrations, however, have been recorded in the vicinity of former DDT manufacturing sites (Reich et al. 1986; West et al. 1994) at levels harmful enough to benthic organisms (Renner 1998; Eganhouse et al. 2000).

The microbiological degradation pathway of DDT in the aquatic environment is quite complex and has not been fully elucidated (Fig. 1), but most of the breakdown products have already been described in the early 1970s (e.g., Jensen et al. 1972). In addition to microbial activity, other agents such as light, catalytic metals, and even higher organisms could either metabolize DDT (e.g., Garrison et al. 2000; Llompart et al. 2003; Van Zwieten et al. 2003) or inhibit its degradation (Gaw et al. 2003).

In the context of ecotoxicological research, however, most studies on DDT contamination and toxicology have focused solely on DDT itself and its main metabolites of the aerobic (DDE, 2,2-*bis*(chlorophenyl)-1,1-dichloroethylene) and anaerobic (DDD, 2,2-*bis*(chlorophenyl)-1,1-dichloroethylene) degradation pathway (e.g., ATSDR 2002). Recently, 2,2-*bis*(chlorophenyl)-1-chloroethylene (DDMU) was also partially included in environmental studies (e.g., Quensen et al. 2001, Xing et al. 2009, Guo et al. 2009). However, other metabolites were neglected in the majority of cases. This trend continued despite the upsurge of recent literature reporting that naturally occurring organisms and/or trace metals in sediments play important roles on the degradation of DDT into metabolites other than DDD, DDE, or DDMU. The occurrence of 2,2-*bis*(chlorophenyl) acetic acid (DDA), 2,2-*bis*(chlorophenyl)benzophenone (DBP), 3,3-*bis*(4-chlorophenyl)-1-propene (DDPU), 1,1-*bis*(4-chlorophenyl)-1-propene (DDPS), and a number of further metabolites have already been documented in several publications (Heberer and Dünnbier 1999; Schwarzbauer et al. 2001, 2003; Kronimus et al. 2006). Authors reported that the detection of DDT metabolites even at lower concentration levels is due to the existence of areas with historical records of high-level industrial emissions and/or agricultural applications. This is exemplified in Table 1 for a contaminated aquatic system (Teltow Canal) within the urban area of Berlin as well as for contaminated sediments of the Mulde River (Germany).

Most of the published toxicological investigations, as mentioned earlier, have focused only on the so-called main metabolites DDD and DDE. As a consequence, although some metabolites have been tested for toxicity (e.g., Planche et al. 1979), no comprehensive toxicological and estrogenicity data for the whole spectra of DDT metabolites are currently available. In order to address this concern, the present study investigated the acute toxicity and biological activities of the following DDT metabolites: *p,p′*-DDMU, *p,p′*-DDMS, *p,p′*-DDCN, and *p,p′*-DDA.

2 Materials and methods

2.1 Chemicals

Four of the more than 17 DDT metabolites which were identified in the Teltow Canal and the Mulde River (Schwarzbauer et al. 2003) were selected based on abundance, water solubility, and position in the catabolism of DDT (Fig. 1). *O,p′*-DDT was used as a reference in all assays. *P,p′*-DDA, *p,p′*-DDMS, *p,p′*-DDMU, and *o,p′*-DDT were obtained from the company Dr. Ehrenstorfer (Augsburg, FRG), *p,p′*-DDCN was freshly synthesised by Schwarzbauer (purity >97–99%; Schwarzbauer 1997).

2.2 Cytotoxicity assay

Cytotoxicity was assayed over 48 h at 20°C by neutral red retention with the permanent cell line RTG-2 derived from the gonads of the rainbow trout (*Oncorhynchus mykiss*; Wolf and Quimby 1962; Borenfreund and Puerner 1984) as described by Kosmehl et al. (2004) with minor modifications. RTG-2 cells were obtained from ICN/Flow (Meckenheim, FRG) and maintained at 20°C in 80 cm^2 plastic culture flasks (Nunc, Wiesbaden, FRG) without additional gassing in Eagle's modification of minimal essential medium supplemented with 20 mM HEPES and Earle's salts, 2 mM L-glutamine, 10% fetal calf serum (Sigma,

Deisenhofen, FRG), and 50 mg/l neomycin sulfate (Sigma). In order to compensate for the moderate biotransformation capacity of RTG-2 cells, DDT metabolites were tested with and without supplementation by S9-preparations from the liver of β-naphtoflavone/phenobarbital-induced rats (CCR, Darmstadt, FRG) (Hollert et al. 2000). *P,p′*-DDA, *p,p′*-DDMS, *p,p′*-DDMU, *p,p′*-DDCN, and *o,p′*-DDT were predissolved in dimethylsulfoxide (DMSO) (maximum DMSO concentration 1%) and tested in six replicates at concentrations between 1.56 and 200 μg/ml.

The viability of the exposed cells was expressed as a percentage of the process controls (i.e., negative controls) and the data were plotted as concentration-response curves. Non-linear regression analysis was performed using SigmaPlot 9.0 (Systat, Erkrath, FRG), and the extract concentrations inducing 50% mortality after 48 h (48 h NR_{50}) were calculated accordingly.

2.3 In vitro EROD assay for dioxin-like activity

The induction of 7-ethoxyresorufin-*O*-deethylase (EROD) activity by *p,p′*-DDA, *p,p′*-DDMS, *p,p′*-DDMU, *p,p′*-DDCN, and *o,p′*-DDT was measured in six replicates in the CYP1A-expressing permanent cell line RTL-W1 derived from rainbow trout liver (*O. mykiss*; Lee et al. 1993) according to Behrens et al. (1998) with slight modifications shown by Wölz et al. (2008). RTL-W1 cells were kindly provided by Drs. Niels C. Bols and Lucy Lee (University of Waterloo, Canada) and cultured at 20°C in 75 cm^2 plastic culture flasks (TPP, Transadingen, Switzerland) in L15 medium supplemented with 9% fetal calf serum and 0.01% penicillin–streptomycin. Prior to exposure to the metabolites, cells were seeded in 96-well plates (TPP) and allowed to attach and proliferate for 72 h. Subsequently, the medium was removed and the cells were exposed for 72 h to the metabolites diluted in medium using eight dilutions. Test concentrations ranged over three orders of magnitude up to levels close to the onset of cytotoxicity [*o,p′*-DDT, 0.05–106.35 μg/ml (0.14–300 μM); *p,p′*-DDMS, 0.0003–256.9 μg/ml (0.001–900 μM); *p,p′*-DDMU, 0.0003–255.2 μg/ml (0.001–900 μM); *p,p′*-DDCN, 0.0002–0.524 μg/ml (0.0008–2 μM); *p,p′*-DDA, 0.0003–0.562 μg/ml (0.001–2 μM)].

As a positive control, 2,3,7,8-tetrachloro-*p*-dibenzodioxin (TCDD, Promochem, Wesel, FRG) was serially diluted to give a final concentration range of 3.13–100 pM on two separate rows of each plate. Induction was terminated by removing the growth medium and freezing at −80°C to lyse the cells. Deethylation of 7-ethoxyresorufin was initiated by adding 7-ethoxyresorufin to each well and incubating in the dark at room temperature for 10 min before the addition of NADPH. The plates were incubated for additional 10 min, and the reaction was stopped by adding fluorescamine dissolved in acetonitrile. EROD activity was measured fluorometrically after another 15 min using a GENios plate reader (Tecan, Crailsheim, FRG; excitation 544 nm, emission 590 nm). Protein was determined fluorometrically using the fluorescamine method (excitation 355 nm, emission 590 nm; Kennedy and Lones 1994, with the protocol detailed in Hollert et al. 2002). The concentration–response curves for EROD induction in the RTL-W1 bioassay were computed by non-linear regression (GraphPad Prism 4, Statcon Software, Witzenhausen, FRG) using the classic sigmoid curve.

Fig. 1 Simplified anoxic degradation pathways of *p,p′*-DDT in aquatic systems (according to Sayles et al. 1997; Zeng and Venkatesan 1999; Eganhouse and Pontolillo 2008; Feng and Turnbull 2009; adapted by Ricking 2010, PhD thesis in preparation). The position of DDCN has only been partially elucidated via the direct pathway from DDT to DBP (Metcalf 1973)

2.4 Endocrine effects in the dot blot/RNAse protection-assay

Estrogenic potentials were tested in a dot blot/RNAse protection-assay with primary hepatocytes from male rainbow trout (Islinger et al. 1999; Smeets et al. 1999).

Tests and Vg1.1 plasmid *P,p′*-DDA, *p,p′*-DDMS, *p,p′*-DDMU, *p,p′*-DDCN, and *o,p′*-DDT were tested in six replicates at non-toxic concentrations of 1, 10, and 50 μM, which had been identified as non-toxic in the acute cytotoxicity assay (see above). As a positive control, 17β-estradiol was used at concentrations of 0.1, 0.2, 1, 2, and 10 nM. To evaluate the anti-estrogenic effectiveness of *p,p′*-DDA, 1 nM 17β-estradiol was added to the test concentrations; combinations of 1 nM 17β-estradiol and 1, 0.1, and 0.01 μM tamoxifen ([Z]-1-[*p*-dimethylaminoethoxyphenyl]-1,2-diphenyl-1-butene, Sigma) were used as reference. The Vg1.1 plasmid was kindly provided by Dr. L. Ren, Medical College Wisconsin, USA (Ren et al. 1996).

Animals Male rainbow trout (*O. mykiss*) weighing 200–500 g were obtained from a local trout farm (Mosbach, FRG) and held in indoor tanks (1,000 l) at a temperature of 12±2°C under flow-through conditions (3 l/min) in purified well water under a 12/12-h dark/light regimen. Fish were fed ad libidum with commercially available trout food and acclimated for at least 4 weeks before use as hepatocyte donors.

Hepatocyte isolation After anaesthesia in a saturated solution of benzocaine (Sigma), the male donor fish was sterilised, opened ventrally, and the liver was pre-perfused and removed carefully. For hepatocyte preparation, the liver parenchyma was digested by collagenase (Roche Diagnostics, Mannheim, FRG), the liver disrupted mechanically, and the hepatocytes filtered and washed three times.

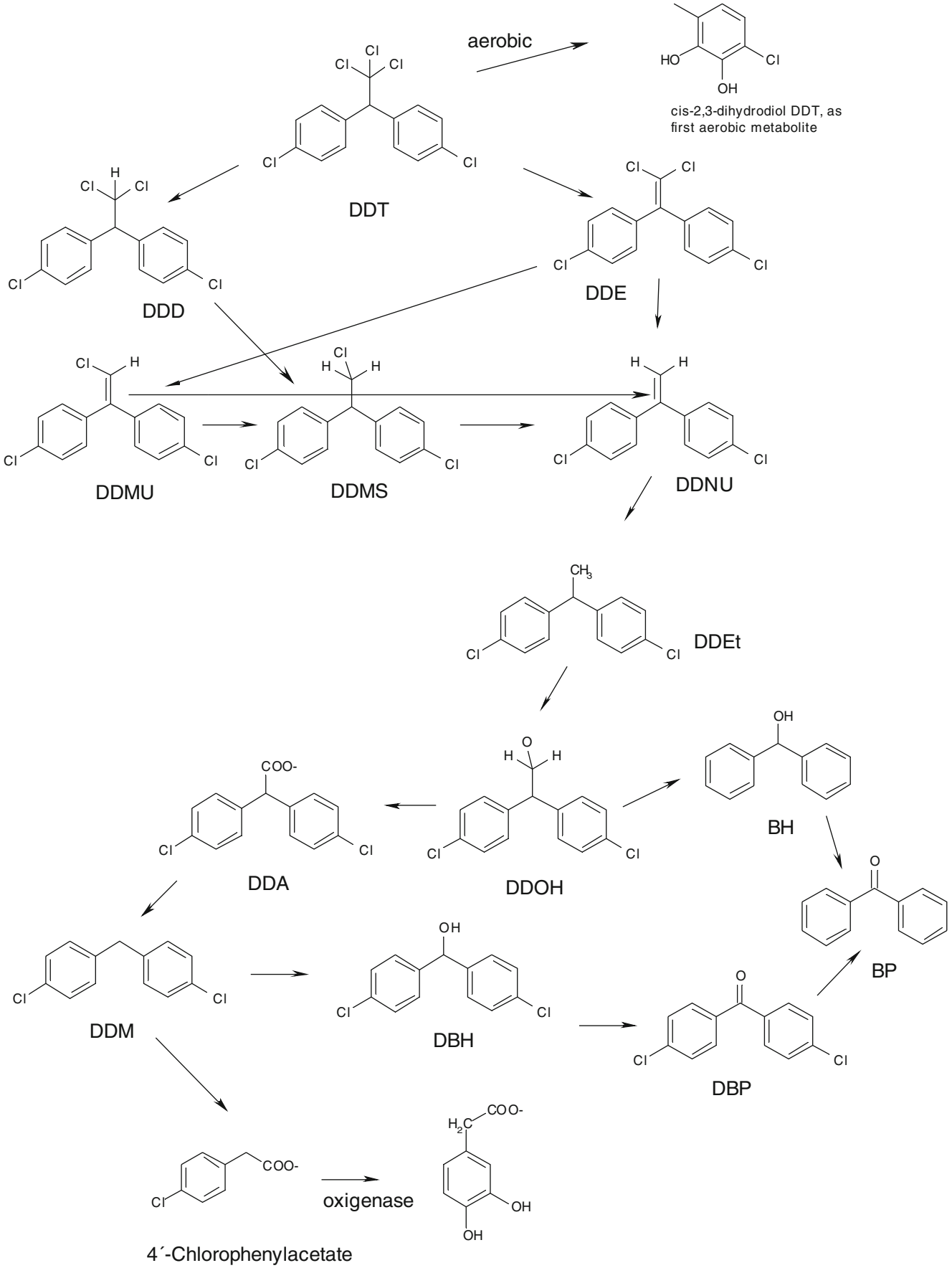
aerobic
cis-2,3-dihydrodiol DDT, as first aerobic metabolite
DDT
DDD
DDE
DDMU
DDMS
DDNU
DDEt
DDA
DDOH
BH
BP
DDM
DBH
DBP
oxigenase
4´-Chlorophenylacetate

Table 1 Occurrence of DDT metabolites in the sediments of selected rivers in Germany

Study area	Fraction	DDT (ng/g)	DDD (ng/g)	DDE (ng/g)	DDX (ng/g)	References
Teltow Canal sediments	Extractable	50–2,570	5,780–64,180	120–1,670	8–33,190 (DDMU, DDMS, DDA, DBP, DDOH, DDCN, DDEt, DDNU)	Heim et al. (2005)
Teltow Canal sediments	Extractable	<5–9,700	9,500–130,000	3,000–13,000	10,000–125,000 (DDMU, DDMS, DDA, DBP, DDOH, DDCN, DDEt, DDNU)	Schwarzbauer et al. (2003)
Teltow Canal sediments	Bound	<20	<20–2,900	<20–500	<20–130,000 (DDMU, DDMS, DDA, DBP, DDOH, DDCN, DDEt, DDNU)	Schwarzbauer et al. (2003)
Mulde River sediments	Extractable	<0.5	<0.5	<0.5–2	<0.5–14	Kronimus and Schwarzbauer (2007)
Mulde River sediments	Bound	<0.5	<0.5	<0.5	<0.5–2	Kronimus and Schwarzbauer (2007)
Mulde River sediments	Extractable	<1–1,100	15–3,500	23–560	9–840 (DDMU, DDMS, DDCN, DBP)	Schwarzbauer (1997)

DBH 2,2-*bis*(chlorophenyl)benzhydrole, *DBP* 2,2-*bis*(chlorophenyl)benzophenone, *DDA* 2,2-*bis*(chlorophenyl)acetic acid, *DDD* 2,2-*bis*(chlorophenyl)-1,1-dichloroethane, *DDE* 2,2-*bis*(chlorophenyl)-1,1-dichloroethylene, *DDEt* 2,2-*bis*(chlorophenyl)ethane, *DDMS* 2,2-*bis*(chlorophenyl)-1-chloroethane, *DDMU* 2,2-*bis*(chlorophenyl)-1-chloroethylene, *DDNU* 2,2-*bis*(chlorophenyl)ethene, *DDOH* 2,2-*bis*(chlorophenyl)-1-ethanol, *DDX* DDT-related compound, *DDCN* 2,2-*bis*(4-chlorophenyl)acetonitrile

Afterwards, the hepatocyte density was adjusted to 1×10^6 cells/ml in M199 culture medium [without phenol red, supplemented with 20 mM HEPES and 1% (*v*/*v*) streptomycin/penicillin solution] and plated into 24-well cell culture plates (Greiner, Frickenhausen, FRG), 2 ml hepatocyte suspension per well, and stored at 14°C (according to Braunbeck and Storch 1992).

Exposure conditions After 24 h of regeneration, 1 ml of the M199 culture medium was replaced by fresh medium containing double-concentrated DDT metabolite dilutions. Final maximum DMSO concentrations were 0.15% and 0.25% (*v*/*v*) in the estrogenic and anti-estrogenic assay, respectively.

Preparation of RNA samples For vitellogenin-mRNA determination in the dot blot/RNAse protection-assay, hepatocytes were harvested after 48 h. Total RNA was prepared using QIA-Shredder and RNeasy Mini-Kits (Qiagen, Hilden, FRG). RNA concentrations and purity were determined by photometry at 260/280 nm, and RNA was stored at −80°C until use.

Dot blot/RNAse protection analysis of vitellogenin-mRNA The intactness of the RNA samples was checked by denaturing agarose/formaldehyde gel electrophoresis and ethidium bromide staining of 28 S and 18 S rRNA. For the analysis of vitellogenin-mRNA, 1 μg of total RNA per sample was blotted onto positively charged nylon membranes (Quibane Nylon+, Qiagen) and treated as described by Zahn et al. (1997) with some modifications by Islinger et al. (1999). Chemoluminescence signals were detected using a Kodak BioMax ML light 1 film at exposure times between 1 and 45 min. The intensities of signals were analysed using E.A.S.Y Win 32 densitometry software (Herolab, Wiesloch, FRG) and compared to those of vitellogenin-mRNA standards. To this end, RNA-T7-transcripts of pGemZf7(−)Vg1.1 were produced with unlabeled nucleotide triphosphates and diluted with TE-buffer (10 mM Tris, 1 mM EDTA, pH 8.0) in a 1:3 dilution series between 300 and 0.14 pg RNA/30 μl. Standard Vg1.1-RNA was stored at −80°C until further use. Data were calculated as relative to 1 nM 17β-estradiol and given as estradiol equivalents.

2.5 Yeast estrogen screen (YES)

Estrogenic potentials were also tested in the YES assay (according to Pawlowski et al. 2004).

Recombinant yeast cells stably transfected with the gene for the human estrogen receptor and containing expression plasmids carrying strong promoter sequences and the lac-Z (β-galactosidase) reporter gene were a gift from Prof. John

Sumpter of Brunel University, UK. YES testing was carried out according to Routledge and Sumpter (1996) using the slightly modification by Rastall et al. (2004). *O,p′*-DDT and the *p,p′*-DDT metabolites were tested over a dose range of 0.1 μM to 20 mM. They were serially diluted along alternate rows of 96 well microtiter plates. A 17β-estradiol (Sigma) positive control was added to a separate row and serially diluted to give a final concentration range of 10^{-8} to 4.80×10^{12} M. One hundred microliters of the ethanol vehicle was then added to each vacant well and the ethanol in all 96 wells was allowed to evaporate. Then, 200 μl of a mixture of 50 ml of YES assay medium containing 500 μl of a 1.65×10^{2} M aqueous solution of the chromogenic substrate chlorophenol-red-β-D-galactopyranoside (CPRG) and 4.0×10^{7} recombinant yeast cells were added to each well. The plates were sealed and incubated at 32°C for 72 h. Estrogenic potentials were determined photometrically at 540 nm following the conversion of CPRG from yellow to red by β-galactosidase secreted into the growth medium in response to the presence of estrogen receptor agonists in the sample.

2.6 Statistics

For the statistical analysis of cytotoxicity, median sample absorption was corrected for median blank absorption and computed as the percentage of the control median. For EC_{50} calculations, medians were plotted in dose–effect curves, while the EC_{50} data were determined using non-linear regression analysis using SigmaPlot 9.0 (Systat, Erkrath, FRG).

Prior to analysis (SigmaStat; SPSS-Jandel, Erkrath, FRG), all data were tested for normality using the Kolmogorov–Smirnov test. When parametric assumptions were met, one-way analysis of variance followed by Dunnett's post hoc test was used to determine which treatments differed significantly from the SCs. In cases where the data or transformed data did not conform to parametric assumptions, a non-parametric Kruskall–Wallis test followed by Dunn's post hoc test was used. Differences were considered significant at $p<0.05$. The no observed effect concentration (NOEC) was the highest concentration showing no significant effects in the post hoc test. The induction equivalence factor (IEF) of the YES was calculated according to the method given by Pawlowski et al. (2004).

3 Results

3.1 Cytotoxicity

All DDT metabolites tested revealed a positive dose–response relationship for cytotoxicity in RTG-2 cells (Fig. 2). The slope of the dose–response curves was quite similar for all metabolites resulting in NR_{50} values from 4 μg/ml (11.3 μM; *o,p′*-DDT) to 80 μg/ml (DDA). S9 supplementation has not changed the toxicity of *o,p′*-DDT significantly. In contrast, comparison of treatments with and without S9-mix showed that cytochrome P450-dependent enzymes caused a clear cut detoxification of *p,p′*-DDMS (27%) and *p,p′*-DDMU (39%). For *p,p′*-DDCN, the cytotoxicity was reduced even to the half (46%) by S9 supplementation (Table 2). Conversely, *p,p′*-DDA and *o,p′*-DDT were not significantly detoxified by S9 supplementation.

3.2 Dioxin-like activities of DDT metabolites

The EROD assay did not detect any dioxin-like activity for all five metabolites when tested in concentrations up to the NOEC of the acute cytotoxicity assay (compare Table 2).

3.3 Estrogenic activity

3.3.1 Dot blot/RNAse protection-assay

The estrogenic potential of the metabolites *p,p′*-DDMS, *p,p′*-DDMU, and *p,p′*-DDCN could be ranked as *o,p′*-DDT > *p,p′*-DDMS > *p,p′*-DDMU > *p,p′*-DDCN (Fig. 3). The highest concentrations of these DDT metabolites (50 μM) stimulated a vitellogenin induction comparable to that of the positive control 1 nM 17β-estradiol. In contrast, *p,p′*-DDA showed a moderate anti-estrogenic effect by competitively inhibiting the estrogenic effect of 17β-estradiol (Fig. 4).

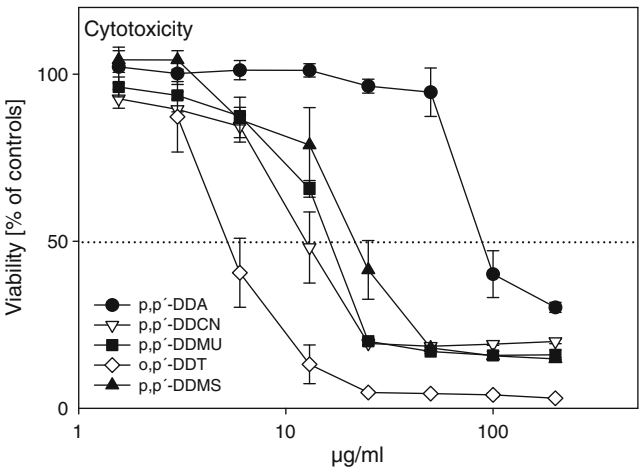

Fig. 2 Acute cytotoxicity of *o,p′*-DDT and the *p,p′*-DDT breakdown products *p,p′*-DDMS, *p,p′*-DDMU, *p,p′*-DDCN, and *p,p′*-DDA in RTG-2 cells without S9 supplementation after 48 h exposure ($n=6$). Cell viability was analysed as neutral red (NR) retention determined photometrically at 540 nm against a reference wavelength of 690 nm. Viability of cells expressed in percent relative to viability of controls

Table 2 NR_{50} values of the acute cytotoxicity assay with and without S9 supplementation as well as the NOEC values without S9 supplementation of *o,p′*-DDT and the *p,p′*-DDT breakdown products *p,p′*-DDMS, *p,p′*-DDMU, *p,p′*-DDCN, and *p,p′*-DDA against the permanent cell line RTG-2 using the neutral red retention assay (n=6)

Substances	NR_{50} with S9 (μg/ml / μM)	NR_{50} without S9 (μg/ml / μM)	NOEC without S9 (μg/ml / μM)
o,p′-DDT	4±1 / 11.3±2.8	4±1 / 11.3±2.8	<2 / 5.6
p,p′-DDMS	28±2 / 98.0±7	22±2 / 77.0±7	8 / 28.0
p,p′-DDMU	28±2 / 98.7±7	17±2 / 60.0±7	8 / 28.2
p,p′-DDCN	28±3 / 106.8±11.4	13±2 / 49.6±7.6	8 / 30.5
p,p′-DDA	86±8 / 305.9±28.5	91±5 / 323.7±17.8	50 / 117.8

DDT 2,2-*bis*(chlorophenyl)-1,1,1-trichloroethane, *DDA* 2,2-*bis*(chlorophenyl)acetic acid, *DDMS* 2,2-*bis*(chlorophenyl)-1-chloroethane, *DDMU* 2,2-*bis*(chlorophenyl)-1-chloroethylene, *DDCN* 2,2-*bis*(4-chlorophenyl)acetonitrile, *NOEC* no observed effect concentration

3.3.2 Yeast assay

In addition to *o,p′*-DDT, two DDT metabolites, *p,p′*-DDMS and *p,p′*-DDMU, displayed dose-dependent activities in the yeast assay (Fig. 5). At this, *p,p′*-DDMU showed only a very weak dose-dependent estrogenic effect with a little toxic effect of the highest concentration. Conversely, *p,p′*-DDCN and *p,p′*-DDA did not display any estrogenic potential (data not shown). At higher concentrations, both *o,p′*-DDT and *p,p′*-DDMU proved to be toxic to the yeast cells. The relative estrogenic potential of the three metabolites in the YES was *o,p′*-DDT > *p,p′*-DDMS >> *p,p′*-DDMU. The estradiol IEF of *o,p′*-DDT was approximately 10^{-6}, when compared to the estrogenic potential of the positive control (17β-estradiol), which is in accordance to other studies.

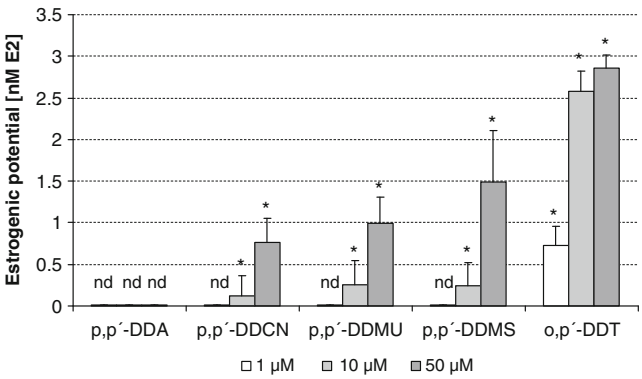

Fig. 3 Estrogenic effects of *o,p′*-DDT and the tested *p,p′*-DDT metabolites in primary hepatocytes from male rainbow trout (n=5). After 48 h of incubation, the induction of Vg-mRNA was assessed using the dot blot/RNAse protection-assay. Data are given as 17β-estradiol equivalents. *P,p′*-DDCN, *p,p′*-DDMU, *p,p′*-DDMS as well as the reference *o,p′*-DDT showed a dose-dependent effect, whereas *p,p′*-DDA did not show any estrogenic effect. * = significant effect in the post hoc test according to Dunnett (p<0.05) when compared to the control; nd = no detectable estrogenic effect

4 Discussion and conclusions

As of this time, a number of studies have already documented the toxic and estrogenic effects as well as ecological relevance of DDT and its breakdown products in a broad variety of animals, plants, fungi, or bacteria (e.g., Planche et al. 1979; Nims et al. 1998; Lotufo et al. 2000; Megharaj et al. 2000). In order to add to existing knowledge concerning the ecotoxicology of DDT and its metabolites, our present study integrated four cell-based assays to elucidate their dioxin-like activity, cytotoxicity, and estrogenic activities. These bioassays provide a rapid, robust, sensitive, and relatively inexpensive alternative to in vivo experiments which are generally time consuming and expensive (Hollert et al. 2005).

The receptor-mediated induction of vitellogenin-mRNA in rainbow trout cells (dot blot/RNAse protection-assay) and the increase in the β-galactosidase expression levels in the yeast cells (YES assay) by *o,p′*-DDT, as shown in the

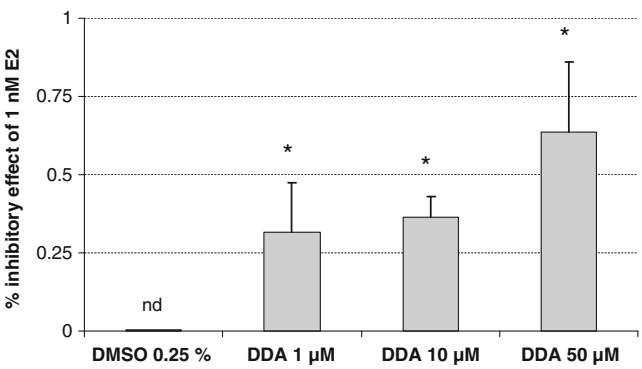

Fig. 4 Anti-estrogenic effect of *p,p′*-DDA as a concentration-dependent inhibition of vitellogenin induction by 1 nM 17β-estradiol in primary hepatocytes from male rainbow trout (n=3). The inhibitory effect is expressed in percentage relative to the inhibitory effect of the positive control, 1 nM 17β-estradiol. * = significant anti-estrogenic effect in the post hoc test according to Dunnett (p<0.05); *nd* no detectable estrogenic effect

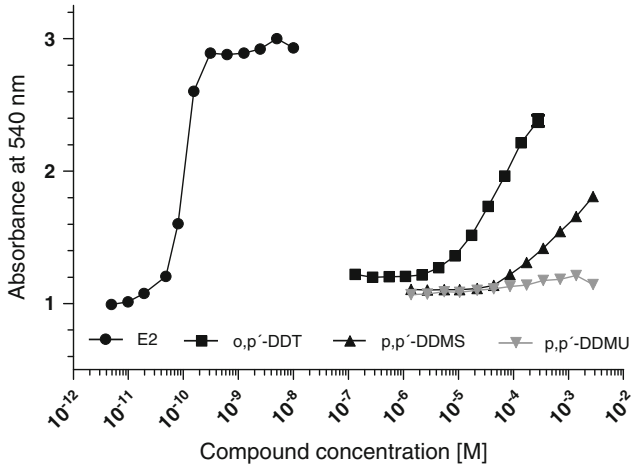

Fig. 5 Concentration-response curves for *o,p′*-DDT, *p,p′*-DDMS, and *p,p′*-DDMU in the yeast estrogen screen (n=3). As a positive control, 17β-estradiol (E2) was used

present study, both provided support for its estrogenic activity. Our findings are likewise in agreement with previous report that *o,p′*-DDT displayed a similar spectrum of estrogenic activity but with less potency than that of the estradiol (Leaños-Castañeda et al. 2007). For instance, we describe in this study a 1,600 to 17,800 times lower estrogenic activity for vitellogenin induction (estrogenic induction equivalence factors, IEFs, of 1.6×10^{-3} to 1.8×10^{-4}) and a 1,000,000 times lower YES activity (IEF=10×10^{-6}), if compared to the 17β-estradiol standard (e.g., Sohoni and Sumpter 1998; Beck et al. 2006). This estrogenicity range was also significantly lower than that of Chen et al. (1997), who reported that an *o,p′*-DDT metabolite could transactivate two yeast expression reporter systems with a 140- to 300-fold lower potency than 17β-estradiol. Meanwhile, the weaker potency of DDT and its metabolites relative to estradiol is correlated to their lower affinity to the estrogen-binding systems in trout liver (Leaños-Castañeda and van der Kraak 2007).

The present study is also the first to report dose-dependent estrogenic potentials of the two DDT metabolites *p,p′*-DDMS and *p,p′*-DDMU in both rainbow trout primary hepatocytes and yeast cells. The potency, however, was shown to be lower than that of the reference *o,p′*-DDT. The probable causes of this seemingly weak estrogenicity could be those processes such as metabolism and degradation of β-galactosidase, associated with long exposure (after 72 h). However, this is unlikely since every concentration (i.e., treatment) we used for all the tested metabolites had uniform incubation time of 72 h. This test was therefore, not time dependent, but rather, dose dependent. Maybe there is a possibility for β-galactosidase degradation to occur, but if this happened, it would have occurred in all samples and would not become obvious since the effect would be integrated in the baseline. A metabolism could happen as well, but in that case, it would have exerted influence even on the lower concentrations, which we were not able to observe in our results. In our opinion, there is a weak dose-dependent estrogenic effect combined with a considerable toxic effect from lowest to the highest concentrations.

One interesting result we found was that, in another metabolite, *p,p′*-DDCN, a weak estrogenic activity was exhibited in the dot blot/RNAse protection-assay, but no reaction, whatsoever, in the yeast estrogen screen (see also Donohoe and Curtis 1996; Petit et al. 1997, 1999). Most likely, the behaviour and consequently, the toxicity of DDT and its metabolites may strongly depend on the specific endpoint measured or the bioassay system used (Binelli et al. 2008).

The ability of *o,p′*-DDT and the *p,p′*-DDT metabolites tested to mimic natural and man-made estrogenic compounds has been linked to their capability to bind and to transcriptionally activate the human estrogen receptor in the same way as previously reported for the major DDT metabolites *o,p′*-DDD and *o,p′*-DDE (Bitman et al. 1968; Chen et al. 1997). Accordingly, these compounds were also shown to induce vitellogenesis in male trout liver cells (Leaños-Castañeda and van der Kraak 2007). Interestingly, however, *p,p′*-DDA was the only DDT metabolite tested in this study that did not induce the receptor-mediated synthesis of vitellogenin mRNA and expression of the β-galactosidase gene. This is in consonance with a previous study by Chen et al. 1997 who reported non-activation and non-binding of *p,p′*-DDA to the human estradiol receptor. Furthermore, our data also suggest that the metabolite has a tendency towards an anti-estrogenic activity as shown by a competitive inhibition of estrogenic effect of the very strong inducer 1 nM 17β-estradiol. In order to prove the relevance of this significant but weak inhibition, future studies have to confirm the anti-estrogenic activity of *p,p′*-DDA by choosing lower concentrations of the inducer E2.

The weak anti-estrogenic behaviour shown by *p,p′*-DDA could be related to the rising polarity of the DDT metabolites in the breakdown cascade and that *p,p′*-DDA seemed to be on the border of estrogenic and anti-estrogenic effect in this respect. Further studies are warranted to elucidate the mechanism of inhibition by *p,p′*-DDA. Since a number of the so-called environmental estrogens were also found to possess an anti-androgenic activity (e.g., DDE, Kelce et al. 1995), it may be of interest to study whether other metabolites display a broader range of endocrine activities (estrogenicity, anti-estrogenicity, androgenicity, and anti-androgenicity) that could clarify their multiple biological effects (Sohoni and Sumpter 1998).

While DDT and its main metabolites were previously shown not to act as Ah-receptor agonists, such properties were not fully investigated to date for the metabolites such as *p,p′*-DDMU, *p,p′*-DDMS, *p,p′*-DDCN, and *p,p′*-DDA, evaluated in this study. These so-called neglected DDT metabolites, just like the parent and main metabolites, belong to the phenobarbital types where they exhibit preferential induction of the 2B subfamily of cytochrome P450 but minimal or no induction of CYP1A (Lubet et al. 1992; Nims et al. 1998). However, this property is up to now quite inconclusive for these least studied metabolites. Futhermore, in other cases, there are marked variations in the behaviour among the different isomers of substances in mechanism-specific bioassays, as recently shown by Preuss et al. (2006, 2010), for different nonylphenol isomers regarding estrogenic properties. This justifies our conduct of further investigation on the dioxin-like activities of related metabolites to bridge the gap between what is known and not yet known. Our study, therefore, offers scientific value by extending the string of information concerning the biological activities of the other less studied metabolites. The present study confirms that *o,p′*-DDT and the least studied *p,p′*-metabolites, namely, DDMU, DDMS, DDA, and DDCN do not show dioxin-like activities in the rainbow trout liver-derived cell line RTL-W1. Though there are inherent variations in these isomers, all of them may lack the typical coplanar structure fitting the CYP1A-binding site for the activation of AhR-receptor and thus failed to induce EROD activity (cf. Petrulis et al. 2001). Another feasible reason could be that, these metabolites may act as transient inducers that bind to the AhR with very low affinity and thus, rapidly degraded by the induced detoxification enzymes (Hilscherova et al. 2000). Other workers (e.g., Jeong and Kim 2002) linked the failure of *o,p′*-DDT to induce EROD activity to contemporary inhibition by its breakdown products. In addition, the ability of *o,p′*-DDT to suppress the TCDD-induced EROD activity in cultured mouse hepatoma cells had been attributed to the decrease of Ah receptor-dependent transcriptional activation caused by the XRE-binding potential of either the nuclear AhR or a transport block of AhR-xenobiotic ligand to the nucleus (Jeong and Kim 2002; Binelli et al. 2006). Nevertheless, further studies should be planned to assess whether the DDT metabolites tested in this study could be inducers of the other subfamily (CYP2B) in the same way as the parent DDT, and the metabolites DDE and DDD.

In contrast to the absence of dioxin-like activity, our data document significant acute cytotoxicity in fish for all DDT metabolites tested. These findings underline the need for their immediate inclusion into risk assessment initiatives in highly contaminated areas. Moreover, our data clearly indicate that both cytotoxicity and estrogenicity of the DDT metabolites decline along the DDT catabolism pathway. This coincides with an increasing level of polarity of the DDT metabolites and therefore, to an increase in their water solubility (DDT 0.003–0.04 mg/l; DDA 11–390 mg/l; Frische et al. 2010). On the one hand, the more polar the DDT metabolites are the higher are their water solubility (higher doses), bioaccessibility, and the metabolism and excretion rates in inner organisms. On the other hand, the more non-polar the DDT metabolites are the lower are their water solubility (lower dose) and bioaccessibility but they are more stable within the organism and may exhibit higher accumulation rates. The dose-dependent and time-related endocrine and cytotoxic cross effects of different simultaneous occurring DDT metabolites on organisms are instantaneously not predictable at all. We therefore recommend further investigations on DDT metabolites, especially with respect to uptake and accumulation in organisms. Additional studies are also warranted to determine the potential additive, synergistic or antagonistic effects that may interfere with basal cytotoxicity and endocrine activities of these neglected metabolites.

The relevance of our results is pertinent and closely mirrored by the natural occurrence of the metabolites investigated. As mentioned before, the knowledge on the environmental concentrations of most of the DDT metabolites are restricted. Beside DDD and DDE, only DDMU has been considered more recently. In particular, investigations on the DDT superfund site at Palos Verdes Shelf, California, USA, demonstrated the importance of DDMU as DDE degradation product with concentrations of up to 5 μg/g in the sediments (Quensen et al. 2001; Eganhouse and Pontolillo 2008). Further on, DDMU has been also detected in estuarine water at Hailing Bay, South China, at low concentrations between 0.05 and 0.2 ng/l (Xing et al. 2009). The occurrence of DDMU in fish species has been reported by Falandysz et al. (2004) and Guo et al. (2009). Only very few studies presented also concentrations of DDM, DBP, or DDA. Wan et al. (2005) reported sediment concentrations of these metabolites up to 50 ng/g in sum at Bohai Bay, China. Only qualitative screening data of DDMS, DDNU, DDCN, and DBP have been given by Franke et al. (2005) for sediments of the Mulde River, FRG. The importance of DDA as a water-related contaminant has been pointed out by Heberer and Dünnbier (1999) with maximum concentrations up to 1,000 ng/l in water of the Teltow Canal, Berlin. Noteworthy, the most comprehensive data set on DDT metabolites has been published for this canal system reporting quantitative data of nearly all known DDT metabolites in the extractable and bound fraction of sediments (Schwarzbauer et al. 2001, 2003). The relative contributions of the so-called neglected DDT metabolites to the total DDX contamination in these sediments reached up to 40% in the free fraction and up to 80% in the reversible bound state, indicating that these metabolites also contrib-

ute significantly to the overall particulate DDT contamination and, consequently, to its ecotoxicological risk. Although the polar DDT metabolites showed a relatively lower ecotoxicological potencies when compared to DDT, we still suggest to include them in the risk assessment if the concentrations have reached levels beyond 100 times greater than those of DDT (as shown, e.g., for the Teltow Canal). Regarding the water phase, the more polar metabolites, in particular DDA, represents the dominant DDX species in pore water, riverine water, and groundwater (Heberer and Dünnbier 1999; Heim et al. 2005; Frische et al. 2010). Hence, an urgent need for a comprehensive ecotoxicological characterisation of all DDT metabolites in the aquatic environment becomes more obvious.

Unfortunately, there remains a paucity of important chemical data needed for risk assessment (e.g., water solubility, K_{ow}, and environmental concentrations) for these DDT metabolites considered in the present study. Conversely, such information is readily available only for the main metabolites like DDD or DDE. As shown in Table 1, DDT metabolite concentrations are always given in terms of total concentrations and not for every single metabolite. The K_{ow} of DDA is the only one mentioned in the literature and in this case it is pH dependent (Jafvert 1990). There is a big gap in chemical and environmental data which has to be filled in before an actual risk assessment could be undertaken. Nevertheless, the results of the present study appear relevant in view of the continued usage of DDT particularly in developing countries (such as in Asia and Africa) and of the continued presence of its metabolite residues worldwide. In fact, technical DDT consists of almost 80% *p,p′*-DDT (e.g., Bitman et al. 1968), and it is *p,p′*-DDT metabolites (i.e., DDMU, DDMS) investigated in this study which are the ones that clearly display cytotoxic and estrogenic activities.

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