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RESEARCH ARTICLE

# Some heterocyclic aromatic compounds are Ah receptor agonists in the DR-CALUX assay and the EROD assay with RTL-W1 cells

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**Abstract**
*Purpose* Heterocyclic aromatic compounds containing nitrogen, sulfur, or oxygen heteroatoms (NSO-HET) have been detected in air, soil, marine, and freshwater systems. However, only few publications are available investigating NSO-HET using in vitro bioassays. To support better characterization of environmental samples, selected NSO-HET were screened for dioxin-like activity in two bioassays.
*Methods* The present study focuses on the identification and quantification of dioxin-like effects of 12 NSO-HET using the DR-CALUX assay, and the 7-ethoxyresorufin-*O*-deethylase (EROD) assay with the permanent fish liver cell line RTL-W1. Changes of the total medium compound concentrations during the test procedure due to, e.g., sorption or volatilization were quantified using GC/MS.
*Results* The NSO-HET benzofuran, 2,3-dimethylbenzofuran, dibenzofuran, dibenzothiophen, acridine, xanthene, and carbazole caused a response in the DR-CALUX assay. Only benzofuran and 2,3-dimethylbenzofuran were also positive in the EROD assay. All other compounds were inactive in the EROD assay. Relative potency (REP) values ranged from $(2.80\pm1.32)\cdot10^{-8}$ to $(3.26\pm2.03)\cdot10^{-6}$ in the DR-CALUX and from $(3.26\pm0.91)\cdot10^{-7}$ to $(4.87\pm1.97)\cdot10^{-7}$ in the EROD assay.
*Conclusions* The REP values were comparable to those of larger polycyclic aromatic hydrocarbons, e.g., fluoranthene and pyrene. Thus, and because of the ubiquitous distribution of heterocyclic aromatic compounds in the environment, the provided data will further facilitate the bioanalytical and analytical characterization of environmental samples towards these toxicants.

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**Keywords** Heterocyclic aromatic compounds · NSO-HET · EROD · DR-CALUX · Ah receptor

## 1 Introduction

Polycyclic aromatic compounds and other tar oil pollutants are a class of ubiquitously distributed environmental contaminants that have been detected in air, soil, marine, and freshwater systems. Industrialized areas, e.g., gasworks and coke manufacturing sites, represent important sources of semisolid tar oil pollutants. Soils and sediments are major sinks of current and historical contaminations with polycyclic aromatic hydrocarbons (PAHs) (Nielsen et al. 1997). Heterocyclic compounds containing nitrogen, sulfur, or oxygen heteroatoms (NSO-HET) simultaneously occur with their unsubstituted PAH analogs. Although NSO-HET constitute not more than 3–13% of total tar oil (Dyreborg et al. 1997; Meyer 1999), up to 40% of the water-soluble fraction consist of these heterocyclic compounds. That leads to increased mobility and bioavailability in the aquatic environment compared to, e.g., PAHs (Licht et al. 1996; Rasmussen and Olsena 2004; Tiehm et al. 2008). Besides processes that involve tar oil, NSO-HET may also originate from dyestuff (Alinsafi et al. 2006; Cripps et al. 1990), pesticides, or pharmaceuticals (Broughton and Watson 2004; Fernández-Alba et al. 2002; Neal and Wu 1994). Although NSO-HET are already known as a substance class of high environmental impact (Barron et al. 2004; Brack and Schirmer 2003), current understanding of environmental fate, distribution, and toxic effects is limited.

Polycyclic substances are known to exhibit a large range of biological effects, e.g., acute toxicity, developmental and reproductive toxicity, mutagenicity, carcinogenicity, or dioxin-like activity (e.g., Brack and Schirmer 2003; Jacob et al. 1984; Robbiano et al. 2004; Zedeck 1980). However, only few publications are available using in vitro bioassays to assess the toxicology of NSO-HET (e.g., Eisentraeger et al. 2008; Sovadinová et al. 2006). Several heterocyclic aromatic compounds are known to be Ah receptor agonists (Brack and Schirmer 2003; Vondrácek et al. 2004) and have been reported or hypothesized to significantly contribute to the overall dioxin-like activity of sediments (Brack and Schirmer 2003; Keiter et al. 2008). Thus, quantitative information about the relative potency of NSO-HET compared to priority pollutants is a prerequisite to enhance the explainability of observations from bioanalytical methods (Sovadinová et al. 2006).

In context of the BMBF (German Federal Ministry of Education and Research) project KORA (retention and degradation processes to reduce contaminations in groundwater and soil), several NSO-HET typically found at creosote-contaminated sites (indole, benzothiophene, benzofuran, 2-methylbenzofuran, 2,3-dimethylbenzofuran, quinoline, 6-methylquinoline, carbazole, dibenzothiophene, dibenzofuran, acridine, and xanthenes; Blotevogel et al. 2008) were investigated using a battery of bioassays (cf. Eisentraeger et al. 2008).

The present study focuses on the identification and quantification of dioxin-like effects of these substances. Specifically, the DR-CALUX assay with pGudLuc1.1 transfected H4IIE rat hepatoma cells (H4IIE-luc) and the 7-ethoxyresorufin-*O*-deethylase (EROD) assay with the permanent fish liver cell line RTL-W1, which is derived from rainbow trout (*Oncorhynchus mykiss*), were used to investigate the relative potency of typical NSO-HET expressed as fixed-effect level-based 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) relative potency factors (REPs). These two assays are widely used for characterization of environmental samples, e.g., sediment extracts (e.g., Keiter et al. 2008). Furthermore, changes of the total medium compound concentrations during the test procedure due to, e.g., sorption, precipitation, or volatilization were quantified using GC/MS. Relative potency factors were calculated with nominal and real concentrations, respectively, and compared.

## 2 Materials and methods

### 2.1 Chemicals

Stock solutions of the used NSO-HET were prepared in dimethyl sulfoxide. Indole (>99%), quinoline (>96%), acridine (>98%), carbazole (approximately 95%), 6-methylquinoline (>98%), benzothiophene (>98%), dibenzothiophene (>98%), benzofuran (>99%), and dibenzofuran (approximately 97%) were purchased from Merck (Darmstadt, Germany). Xanthene (99%) was supplied by Sigma-Aldrich (Deisenhofen, Germany), 2-methylbenzofuran (≥95%) and 2,3-dimethylbenzofuran (≥95%) were purchased from Fluka (Buchs, Switzerland).

### 2.2 DR-CALUX assay

The DR-CALUX assay utilizes the rat hepatoma cell line H4IIE, which has been stably transfected with the pGudLuc1.1 luciferase reporter gene construct (Garrison et al. 1996; Murk et al. 1996). The sensitivity to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, expressed as the effective concentration causing 50% of the maximum effect level ($EC_{50}$) was 15.4±3.8 pM (mean value ± standard deviation, $n$=36) in the present study, while others reported values of 12 and 14 pM, respectively (Long et al. 2003). DR-CALUX was conducted according to the protocols recently published elsewhere (Olsman et al. 2007; Wölz et al. 2008).

Cells were obtained from Biodetection Systems (Amsterdam, Netherlands) and cultivated at 37°C in minimal essential medium (α-MEM) supplemented with 10% (*v*/*v*) fetal bovine serum (FBS). The cells were seeded into 96-well plates 24 h prior to exposure and allowed to reach 100% confluence. Subsequently, sample dilutions were prepared in culture medium and added to the cells in triplicate wells. The chemicals were tested in 12 nominal concentrations, while the highest concentration was chosen to be non-cytotoxic as determined using the neutral red retention assay (Wölz et al. 2008). In each assay, a concentration series of 0-300 pM 2,3,7,8-TCDD (Accustandard, New Haven, USA) was included. After 24 h exposure at 37°C, the culture medium was discarded; cells were washed twice with phosphate-buffered saline (PBS) and lysed in PBS at −20°C overnight. Subsequently, the luciferase activity was measured using the Luclite assay kit (PerkinElmer, Upplands Väsby, Sweden) with addition of the substrate luciferin, and after incubation for 1 h in darkness at 20°C. Cell lysates were transferred to white 96-well microplates and luminescence was determined in a Wallac 1420 Victor$^2$ multiwell-plate reader (PerkinElmer, Upplands Väsby, Sweden).

### 2.3 EROD assay

Induction of 7-ethoxyresorufin-*O*-deethylase was measured in the CYP1A expressing permanent fish liver cell line RTL-W1 (from rainbow trout, *O. mykiss*) with slight modifications to a method previously published elsewhere (Behrens et al. 1998; Gustavsson et al. 2004). RTL-W1 cells (Lee et al. 1993) were obtained from Drs. Niels C. Bols and Lucy Lee, University of Waterloo, Canada. The sensitivity to 2,3,7,8-TCDD, expressed as the $EC_{50}$ was 12.7±3.4 pM (mean value ± standard deviation, $n$=36) in the present study, while other researchers reported 6±2.6 pM (Clemons et al. 1997). Cells were cultured at 20°C in Leibovitz L15 medium supplemented with 8% (*v*/*v*) FBS and 1% (*v*/*v*) penicillin/streptomycin solution (Gustavsson et al. 2004). Prior to exposure, cells were seeded into 96-well microplates and grown to 100% confluence over 72 h at 20°C. Subsequently, the medium was removed and cells were exposed to the samples in eight nominal concentrations (six replicates per concentration) for 48 h at 20°C in a refrigerated incubator (Memmert, Schwabach, Germany). The highest concentration was chosen to be non-cytotoxic as determined using the neutral red retention assay (Wölz et al. 2008). A dilution series of 0–100 pM 2,3,7,8-TCDD (Promochem, Wesel, Germany) was included in duplicates on each plate. Exposure was terminated by removing the growth medium and cells were subsequently lysed at −70°C. Plates were thawed and 100 µl of 1.2 µM 7-ethoxyresorufin were added to each well before deethylation was initiated with 0.09 µM NADPH in phosphate buffer (pH 7.8). The reaction was stopped after 10 min by adding 100 µl of 0.54 mM fluorescamine in acetonitrile. The deethylation of exogenous 7-ethoxyresorufin was measured fluorometrically (excitation 544 nm, emission 590 nm) using a GENios plate reader (Tecan, Crailsheim, Germany). Whole protein was determined fluorometrically using the fluorescamine method (excitation 355 nm, emission 590 nm; Brunström and Halldin 1998; Hollert et al. 2002).

### 2.4 Relative potency factor calculations

Concentration–response curves in the EROD assay and the DR-CALUX, respectively, were calculated with the software GraphPad Prism 4.0 (GraphPad, San Diego, CA, USA) using the logistic Hill equation model. The bottom value of the equation was set to the response of solvent controls. Fixed-effect level-based REP values (Brack et al. 2000) were calculated as follows:

$$\mathrm{REP} = \frac{\mathrm{EC}_{25,\mathrm{TCDD}}(\mathrm{TCDD})}{\mathrm{EC}_{25,\mathrm{TCDD}}(\mathrm{chemical})}$$

Effect concentrations ($EC_{25,TCDD}$) refer to the concentration of the substance causing 25% of the maximum effect level of 2,3,7,8-TCDD. Within all REP calculations the $EC_{25}$ value for 2,3,7,8-TCDD, $EC_{25,TCDD}$(TCDD), was always derived from the same plate as the sample $EC_{25,TCDD}$.

### 2.5 Chemical analysis

To study the effects of, e.g., sorption to plastic plates or precipitation on actual total medium NSO-HET concentrations compared to nominal concentrations, 96-well microplates were prepared in the same way as for the EROD assay without adding cells. Before and after 48 h incubation, each solution was removed from the plate and stored in a glass vial with PTFE cap. The NSO-HET were extracted (liquid–liquid extraction) with methyl-*tert*-butyl ether and subsequently analyzed using gas chromatography (Agilent technologies GC 6890 N). The GC was equipped with an autosampler (Agilent technologies) and mass selective detector (Agilent technologies MS 5973 Network) operated in Single Ion Monitoring mode. The limit of detection was 0.1 µg $L^{-1}$ for the investigated substances (Figs. 1 and 2).

## 3 Results and discussion

In both assays, the results were used to construct concentration–response curves (Figs. 1, 3, and 4) from which REP values were calculated. The efficacy of the investigated substances ranged from very weak to close to that of

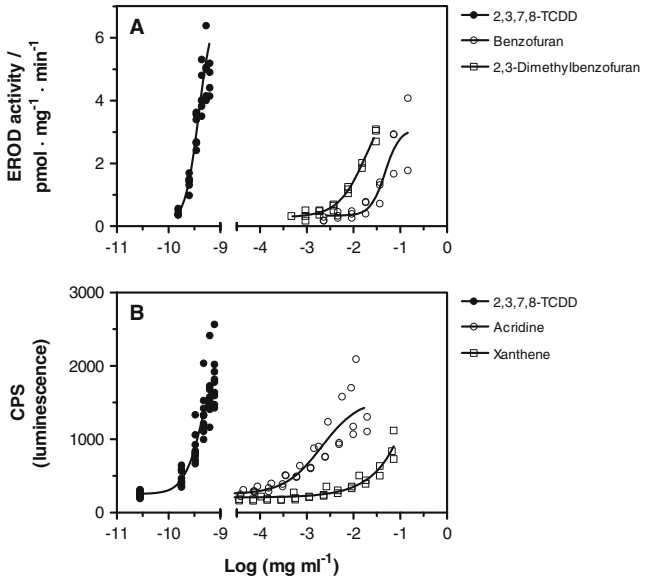


**Fig. 1** Representative concentration–response curves measured in single experiments in the EROD assay and the DR-CALUX, respectively. **a** Shows the curves for the most potent inducers in the EROD assay (benzofuran and 2,3-dimethylbenzofuran), and **b** for the DR-CALUX (acridine and xanthene). The concentration–response curves for 2,3,7,8-TCDD are given for reference. Concentration values on the *x*-axis refer to nominal medium concentration of the substances. *Dots* represent the values from independent replicate measurements

2,3,7,8-TCDD (e.g., Fig. 1). Although most of the compounds, however, significantly induced EROD and luciferase at least at higher concentrations (one-way ANOVA with Dunnett's post-hoc test, $p \leq 0.05$), only benzofuran and 2,3-dimethylbenzofuran reached 25% of the maximum induction by the control substance 2,3,7,8-TCDD in the EROD assay. Indole, quinoline, 6-methylquinoline, 2-methylbenzofuran, and 1-benzothiophene did not reach 25% of the maximum induction of TCDD in the DR-CALUX assay. Consequently, it was possible to calculate REP values in the respective bioassay only for substances exceeding 25% of the maximum induction of TCDD in at least two replicates (Table 1, Fig. 2). Since the temporal response kinetics of the cells were not assessed in the current study, the comparably low efficacies of some of the substances might be attributed to metabolism and a resulting transient induction of EROD or luciferase. A number of authors have demonstrated that maximum induction of several readily metabolized AhR ligands was observed within a few hours after dosing (e.g., Clemons et al. 1997; Louiz et al. 2008). Thus, future studies on NSO-HET should address this point.

Acridine was the most potent AhR agonist in the DR-CALUX assay, whereas 2,3-dimethylbenzofuran was the most potent EROD inducer. Each substance was tested independently at least three times in both tests, since values from both assays showed high variation. For example, for the DR-CALUX assay with dibenzofuran, the relative

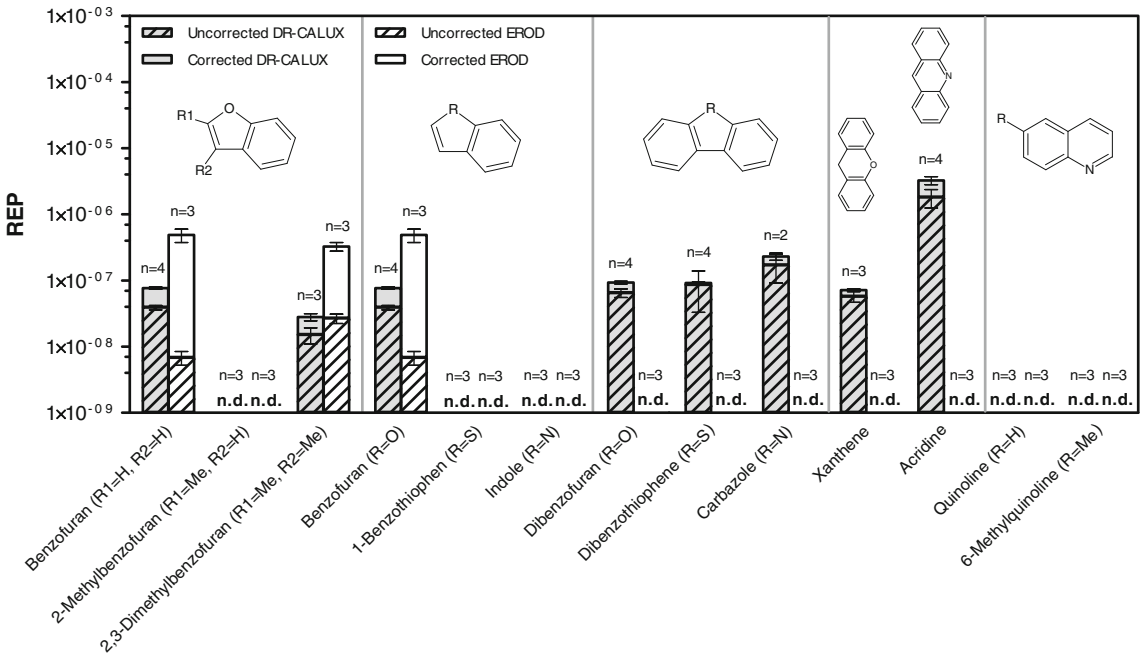


**Fig. 2** Uncorrected (referring to nominal concentrations) and corrected (referring to measured total medium concentration) relative potencies (*REPs*) of the investigated heterocyclic compounds relative to 2,3,7,8-TCDD as measured in the EROD and the DR-CALUX assay. *Bars* represent mean values ± standard error of the number of *n* valid replicate measurements stated *above the bars*. All substances were tested at least three times in the respective test system. *n.d.* inactive in assay system, i.e., substances which did not reach 25% induction of the 2,3,7,8-TCDD standard

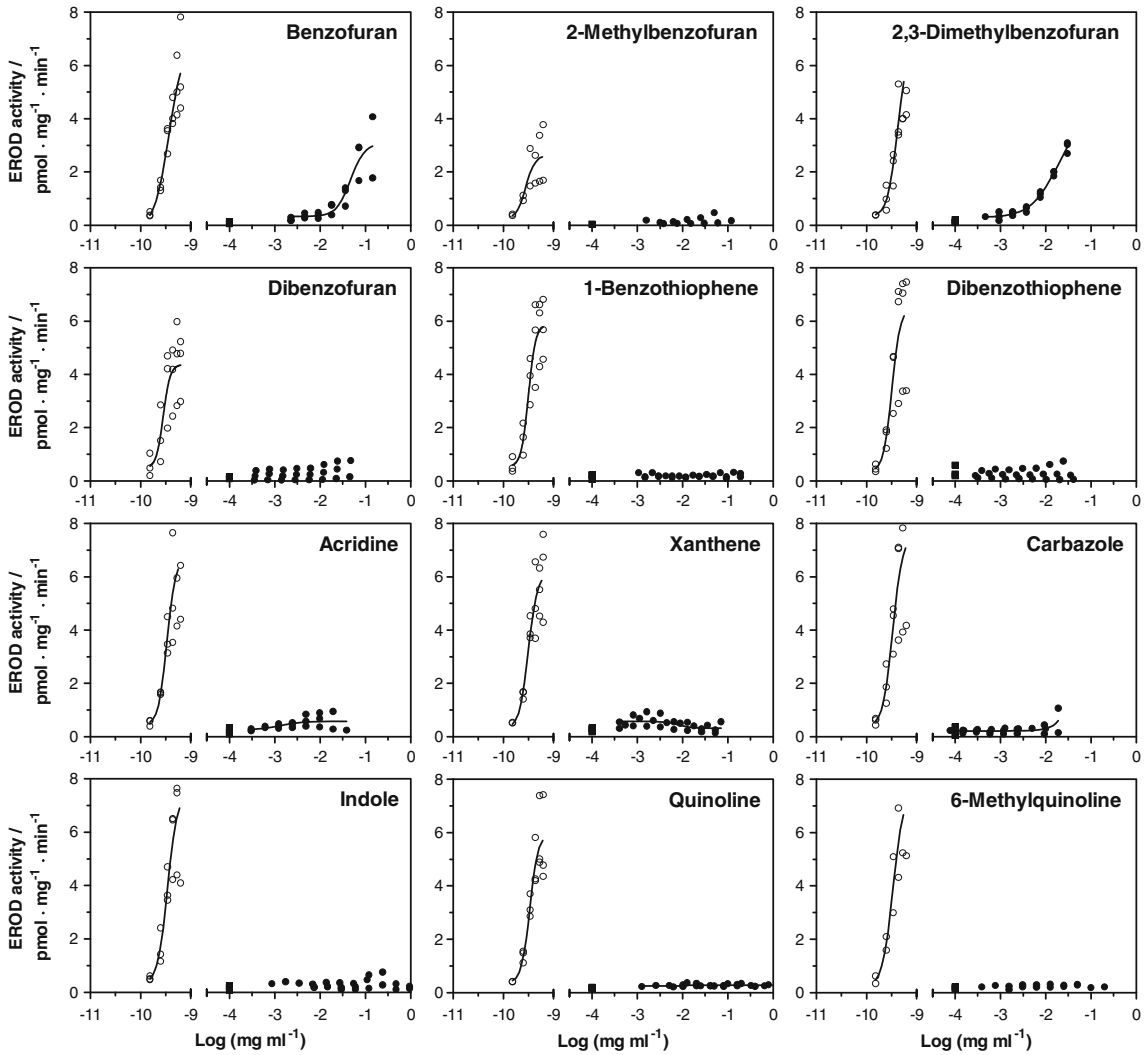


**Fig. 3** Dose–response curves in the EROD assay for all investigated heterocyclic compounds (*closed circles*), as well as standard curves for 2,3,7,8-TCDD (*open circles*) and control values (*filled squares*). Concentration values on the *x*-axis refer to nominal medium concentrations of the substances. *Dots* represent the values measured in independent replicate experiments

standard deviation (RSD) averaged approximately 43%. Specifically, reproducibility in the EROD assay was comparably low. The inter-assay variation of TCDD data, however, was 24.7% (RSD) in the DR-CALUX and 26.7% (RSD) in the EROD assay, indicating that the high variation of values for NSO-HET is more likely due to the chemical properties (low efficacy and potency) rather than to the experimental procedures. However, also Keiter et al. (2009) reported using a data set with 59 independent investigations that the variability of EROD assay results is approximately ±35%.

Losses of the substances due to, e.g., sorption, precipitation, and volatilization were shown to be an issue of concern by chemical analyses. The loss of substance from the culture medium exceeded 90% for benzothiophene, benzofuran, 2-methylbenzofuran, and 2,3-dimethylbenzofuran after 48 h. Furthermore, carbazole, dibenzofuran, acridine, and xanthene showed losses up to approximately 90%. A comparison of the 96-well plates that were used in this test with the same plates sealed with a semi-permeable membrane (Renner, Dannstadt, Germany) showed no significant differences (details not shown). For example, the loss of benzothiophene, benzofuran, 2-methylbenzofuran, and 2,3-dimethylbenzofuran was slightly reduced to <90%, but sealing did not change the results of the EROD assays (data not shown). Hence, it seems likely that the loss of substance is due to sorption of the highly non-polar NSO-HET onto the surface of the wells or precipitation of the compounds in culture medium due to their comparably low solubility (cf. Eisentraeger et al. 2008). These processes decrease the actual concentration of a substance and could possibly lead to an underestimation of its environmental potency. Similar effects have been observed in the algae growth inhibition and the *Daphnia magna* immobilization tests (Eisentraeger et al. 2008). To correct for these losses in the assessment (Table 1), correction factors were applied that

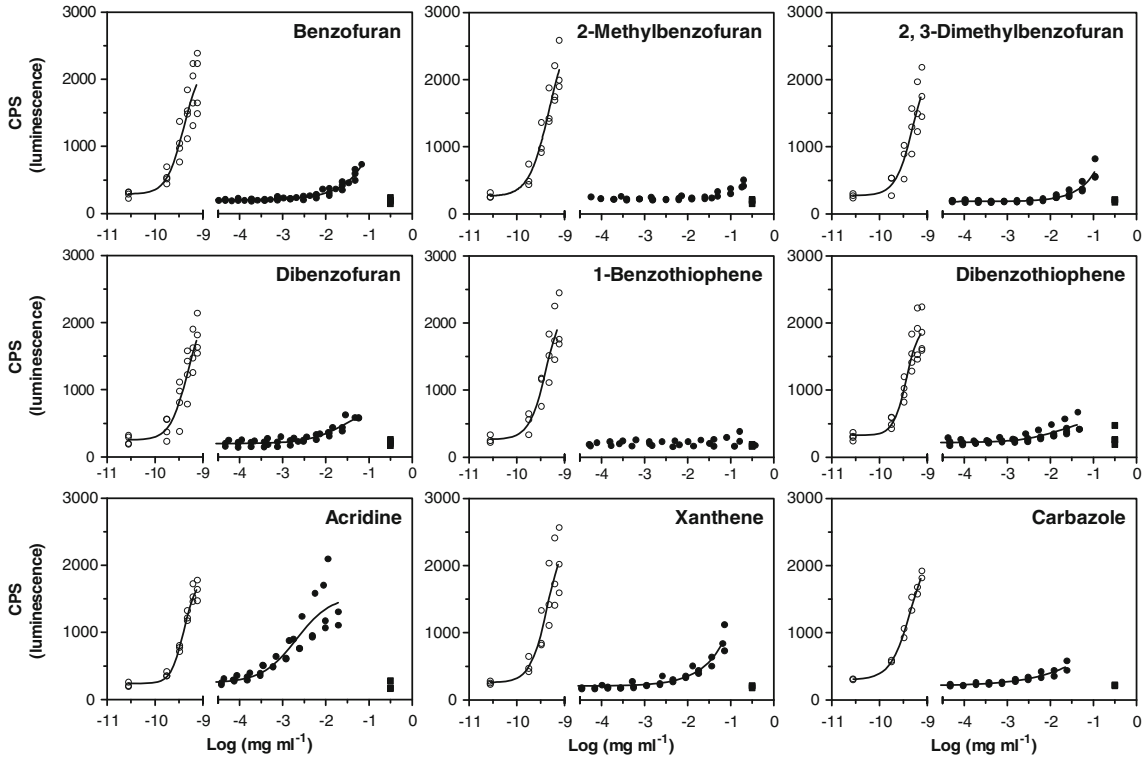


**Fig. 4** Dose–response curves in the DR-CALUX assay for the investigated heterocyclic compounds (*closed circles*), as well as standard curves for 2,3,7,8-TCDD (*open circles*) and control values (*filled squares*). Concentration values on the *x*-axis refer to nominal medium concentrations of the substances. *Dots* represent the values measured in independent replicate experiments

were based on the chemically measured effective concentrations. Linear adsorption kinetics were assumed and losses in the DR-CALUX (24 h incubation) were interpolated from the measured concentrations in the EROD assay (48 h incubation). By multiplying the nominal concentration in the test with the resulting correction factor, the REP values change proportionally. This correction did not drastically change the relative potencies in comparison to the other

**Table 1** Corrected REPs (mean value ± standard deviation) and respective corrected $EC_{25}$ values (mean value ± standard deviation) of the investigated heterocyclic compounds relative to 2,3,7,8-TCDD in both the DR-CALUX and the EROD assay, and losses in the EROD assay used for the calculation of corrected REPs, respectively

| Substance | REP DR-CALUX | $EC_{25}$ DR-CALUX/μg ml$^{-1}$ | REP EROD assay | $EC_{25}$ EROD/μg ml$^{-1}$ | Loss EROD/% |
|---|---|---|---|---|---|
| Benzofuran | $(7.70\pm1.05)\cdot10^{-8}$ ($n$=4) | 20.65±0.10 ($n$=4) | $(4.87\pm1.97)\cdot10^{-7}$ ($n$=3) | 0.56±0.17 ($n$=3) | 98.6 |
| 2-Methylbenzofuran | n.d. ($n$=3) | n.d. ($n$=3) | n.d. ($n$=3) | n.d. ($n$=3) | 99.7 |
| 2,3-Dimethylbenzofuran | $(2.80\pm1.32)\cdot10^{-8}$ ($n$=3) | 43.99±8.33 ($n$=3) | $(3.26\pm0.91)\cdot10^{-7}$ ($n$=3) | 0.90±0.50 ($n$=3) | 91.8 |
| Dibenzofuran | $(9.35\pm2.74)\cdot10^{-8}$ ($n$=4) | 8.84±3.77 ($n$=4) | n.d. ($n$=3) | n.d. ($n$=3) | 61.1 |
| 1-Benzothiophene | n.d. ($n$=3) | n.d. ($n$=3) | n.d. ($n$=3) | n.d. ($n$=3) | 97.2 |
| Dibenzothiophene | $(9.17\pm11.32)\cdot10^{-8}$ ($n$=4) | 11.02±5.73 ($n$=4) | n.d. ($n$=3) | n.d. ($n$=3) | 11.4 |
| Acridine | $(3.26\pm2.03)\cdot10^{-6}$ ($n$=4) | 1.72±0.57 ($n$=4) | n.d. ($n$=3) | n.d. ($n$=3) | 88.6 |
| Xanthene | $(7.17\pm2.29)\cdot10^{-8}$ ($n$=3) | 29.14±6.53 ($n$=3) | n.d. ($n$=3) | n.d. ($n$=3) | 40.2 |
| Carbazole | $(2.31\pm1.51)\cdot10^{-7}$ ($n$=2) | 1.88±1.26 ($n$=2) | n.d. ($n$=3) | n.d. ($n$=3) | 51.9 |
| Indole | n.d. ($n$=3) | n.d. ($n$=3) | n.d. ($n$=3) | n.d. ($n$=3) | n.a. |
| Quinoline | n.d. ($n$=3) | n.d. ($n$=3) | n.d. ($n$=3) | n.d. ($n$=3) | n.a. |
| 6-Methylquinoline | n.d. ($n$=3) | n.d. ($n$=3) | n.d. ($n$=3) | n.d. ($n$=3) | n.a. |

*n.d.* inactive in assay system, i.e., substances which did not reach 25% induction of the 2,3,7,8-TCDD standard

NSO-HET (Fig. 2), but should be considered as an attempt to reconstruct the maximum potency of each substance.

Benzofuran and 2,3-dimethylbenzofuran showed exceptional properties. First, both were the only compounds which were positive in the EROD assay with RTL-W1 cells. Second, the potency of both compounds in the EROD assay was higher compared to the DR-CALUX assay. Thus, the mechanism of EROD induction should be further investigated in future studies. Furthermore, both substances are volatile and it can be supposed that these substances are at risk for getting lost in sediment extraction processes that involve heat. Since both compounds were the only effective EROD inducers out of the NSO-HET tested, dioxin-like activity of sediment extracts obtained from those extraction procedures, e.g., Soxhlet extraction, should be investigated critically.

In summary, NSO-HET constitute a group of weak to moderate Ah receptor agonists and EROD inducers (Table 1). PAHs of about the same size (two to three aromatic rings) as the investigated substances, however, e.g., naphthalene, fluorene, and phenanthrene, are not known to cause an induction (Barron et al. 2004). The REP values were comparable to those of larger PAHs (four to five aromatic rings), e.g., fluoranthene ($2 \cdot 10^{-9}$) and pyrene ($3.85 \cdot 10^{-7}$; Barron et al. 2004). Results indicate that the NSO-HET group of chemicals should be accounted for in bioanalytical and chemical analysis-based mass balance calculations, since they may be involved in the often encountered unexplained AhR agonistic activity of environmental samples (Andersson et al. 2009; Keiter et al. 2008). With respect to the high mobility of NSO-HET in the environment, their toxicity, and persistence, the results of this study further indicate that NSO-HET should be regarded as highly relevant in the context of environmental risk assessment.

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