



In vitro assessment of AhR-mediated activities of TCDD in mixture with humic substances

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ABSTRACT

Humic substances (HS) are ubiquitous natural products of decomposition of dead organic matter. HS is present in most freshwaters at concentrations ranging from 0.5 to 50 mg L⁻¹. Organic carbon can represent 20% dry weight of sediments. Recently, the interaction of dissolved HS with the aryl hydrocarbon receptor (AhR) has been demonstrated. The AhR is a cytosolic receptor to which persistent organic pollutants (POPs) can bind and many of their toxic effects are mediated through interactions with this receptor. We describe *in vitro* effects (using H4IIE-*luc* cells) of binary mixtures of various HS with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), since in contaminated environments these compounds occur simultaneously. Six out of 12 HS samples activated AhR even at environmentally relevant concentrations (17 mg L⁻¹), but did not reach the full AhR-activation even at excessive concentration. In simultaneous exposure of H4IIE-*luc* to HS (17 mg L⁻¹) and TCDD (1.2 pM) without any preincubation prior to exposure, either significant additive or facilitative effects were observed. No negative interactions, due to possible sorption of TCDD to HS was observed. Nevertheless, if the HS–TCDD binary mixture was preincubated for 6 days prior to the exposure on H4IIE-*luc* cells, the additive and facilitative effects were less due to possible sorption of TCDD onto HS. Similar results were obtained from analogous experiments with greater concentrations of both TCDD and HS.

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1. Introduction

Humic substances (HS) are ubiquitous natural products of decomposition of dead organic matter. Generally, HS can be divided into three operationally defined groups: humic acids (HA), fulvic acids (FA) and humins. In the aquatic environment, HS form approximately 50–70% of dissolved/natural organic matter (D/NOM) (Timofeyev et al., 2004), which is present in most natural freshwaters at concentrations ranging from 0.5 to 50 mg L⁻¹. Organic carbon can comprise as much as 20% dry weight in sediments (Steinberg, 2003; Akkanen et al., 2005). Biological effects of HS can be both direct and indirect. Indirect effects such as modulating the availability of toxicants or nutrients via interactions with various HS structures have long been known. However, more recently, it has been found that exposure to HA can also lead to direct biochemical and physio-

logical effects such as changes of heat shock proteins expression and biotransformation enzymes activity, or hormone-like effects in animals (Steinberg et al., 2008; Menzel et al., 2005). Furthermore, humic substances can facilitate life in extreme aquatic environments, and have the potential to extend lifespan, as was shown for nematode *Caenorhabditis elegans* (Steinberg et al., 2007). In a previous *in vitro* study, we described the interaction of HS with the aryl hydrocarbon receptor (AhR) (Janosek et al., 2007), which has been also confirmed *in vivo* (Matsuo et al., 2006). In the environment, especially in sediments, soils and water, persistent organic pollutants (POPs) such as e.g. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are expected to occur in the presence of naturally occurring HS. With regards to the newly recognized properties of some HS (e.g. ability to activate AhR), in combination with other HS properties (e.g. sorption capacity, tenside-like action, etc.), we can not easily predict the resulting ecotoxicity of HS–TCDD mixture. In this paper, we describe *in vitro* effects of binary mixtures of various HS with TCDD on AhR-activation and discuss the possible mechanisms of interaction.

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2. Material and methods

HS isolated from different matrices were purchased from Fluka (Switzerland) – HA-Fluka, Sigma Aldrich (Czech Republic) – HA sodium salt, Humintech GmbH (Germany) – HuminFeed, and International Humic Substances Society (IHSS, USA) – remaining nine HS samples. For the purposes of the study upon which we report here, two NOM samples (IHSS) are included in the group of HS. However, we are aware that in practice HS are a subgroup of NOM. HS were dissolved in 0.05 M NaOH to achieve final concentrations 17, 50, 150 and 300 mg L⁻¹. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from Dr. Ehrenstorfer (Germany). Experiments were carried out using TCDD dissolved in ethanol.

H4IIE-*luc* (rat hepatocarcinoma) cells stably transfected with the luciferase gene under control of AhR were used for determination of AhR-activation. Cells were grown and maintained in DMEM medium containing 10% fetal calf serum (both PAA laboratories, Austria) at 5% CO₂ and 37 °C. Once the cells reached about 70% confluence they were passaged and seeded into a sterile 96-well plate at density 15,000 cells/well. After 24 h, the cells were exposed in triplicate to the tested samples (17, 50, 150 and 300 mg L⁻¹ HS) or reference compound (dilution series 0.1–100 pM TCDD) or mixtures of TCDD and HS (1.2 pM TCDD with 17 mg L⁻¹ HS, 3.7 pM TCDD with 50 mg L⁻¹ HS, and 500 pM TCDD with 150 mg L⁻¹ HS) for 24 h at 37 °C (final vehicle concentration was 0.5% v/v). Cells exposed to DMEM with 0.5% EtOH and 0.5% 0.05 M NaOH were used for the appropriate vehicle controls. Intensity of luciferase luminescence was measured using Promega Steady Glo Kit (Pro-

mega, Germany) after 24 h exposure. Three independent assays have been conducted for each concentration tested, except the lowest HS (17 mg L⁻¹) and TCDD (1.2 pM) concentration where six independent assays have been conducted. More repetitions of the tests with the last mentioned samples were conducted because these were the most environmentally relevant than the greater concentrations tested. Assessment of AhR-mediated activity of mixtures at greater concentrations (TCDD ~ 3.7 pM and HS ~ 50 mg L⁻¹) was conducted to examine the effects of mixtures at concentrations with greater AhR-responses. Assessment of AhR-mediated activity of excessive concentrations of both HS (150 mg L⁻¹) and TCDD (500 pM; AhR saturating concentration), which are environmentally unrealistic, was conducted to examine HS ability to act as partial agonist/antagonist of AhR.

All samples (HS alone, TCDD alone, and the three HS–TCDD binary mixtures described above) were added to the medium over cells either immediately, or after preincubation. Preincubation was carried out in cultivating medium supplemented with 1% Gentamicin by shaking in glass vials in the dark for the duration of 1, 4, 24 and 144 h.

Such paired experimental designs (non-incubated samples, and the analogous samples preincubated for 1, 4, 24 or 144 h prior to exposure on H4IIE-*luc* cells) was performed to model several situations that can occur in real environmental mixtures. At the time of accidental releases of POPs into the environment, organisms are exposed to fresh mixtures of HS and POPs (plus other various compounds). This situation was simulated by exposure of H4IIE-*luc* to non-preincubated HS–TCDD mixtures. Nevertheless, during the

Table 1
Summary of AhR-mediated activity of 12 HS samples at concentrations 17, 50 and 150 mg L⁻¹, TCDD at concentrations 1.2, 3.7 and 500 pM, and corresponding binary mixtures of HS and TCDD. The table shows results for each group of samples measured both immediately after mixing and after the 6 day preincubation.

	AhR-mediated activity (% of TCDD _{max} induction)											
	TCDD ~ 1.2 pM, HS ~ 17 mg L ⁻¹				TCDD ~ 3.7 pM, HS ~ 50 mg L ⁻¹				TCDD ~ 500 pM, HS ~ 150 mg L ⁻¹			
	no preinc.		6-day preinc.		no preinc.		6-day preinc.		no preinc.		6-day preinc.	
	%	SD	%	SD	%	SD	%	SD	%	SD	%	SD
TCDD	16.3	1.2	15.2	1.2	38.5	3.8	39.2	2.0	100.0	18.5	100.0	4.76
HuminFeed	8.4	0.6*	8.5	1.2*	21.0	1.8*	28.4	2.8*	62.9	6.0*	60.7	3.8*
HuminFeed + TCDD	25.6	4.9*	20.7	5.3	57.2	11.8*	47.8	7.4*	110.2	11.7	119.2	20.8
HA (Fluka)	13.1	0.4*	15.1	1.2*	27.8	0.3*	45.7	3.7*	72.9	5.3*	74.2	3.8
HA (Fluka) + TCDD	28.3	5.4*	24.9	4.4*	56.4	7.8*	54.9	7.8*	81.1	22.8	96.2	10.1
Elliott Soil HA	13.9	0.4*	11.5	1.0*	31.2	1.0*	35.2	2.2*	78.5	1.8*	64.8	3.8*
Elliott Soil HA + TCDD	31.4	8.6*	21.9	6.8	58.8	8.4*	48.3	14.2	116.1	25.6	110.9	8.3
Florida Peat HA	7.3	0.1*	6.0	1.2*	13.1	0.5*	18.5	2.5*	51.1	1.8*	37.6	2.0*
Florida Peat HA + TCDD	23.7	3.6*	18.0	3.9	54.3	5.6*	44.3	13.0	135.9	14.5*	137.5	17.7*
Leonardite HA	12.7	0.7*	9.5	0.8*	30.4	2.1*	34.9	2.8*	64.0	3.2*	56.4	4.3*
Leonardite HA + TCDD	28.7	6.4*	26.5	9.5	57.0	7.8*	55.1	21.8	89.1	17.2	99.9	9.7
HA sodium salt (Aldrich)	8.9	0.3*	9.6	1.9*	39.6	2.5*	38.7	2.9*	52.3	6.3*	84.1	8.6*
HA sod. salt (Aldrich) + TCDD	26.4	6.7*	22.6	5.0*	55.7	7.0*	51.7	8.5*	112.4	27.0	112.8	10.1
Suwannee River HA	3.1	0.6*	2.4	1.0	0.7	0.6	7.0	1.7*	5.9	2.2*	9.0	1.5*
Suwannee River HA + TCDD	20.2	2.4*	17.9	3.3	48.0	5.3*	46.3	11.0	120.3	18.2	108.1	14.6
Waskish Peat HA	2.0	0.8	2.8	0.8*	1.5	1.4	8.5	3.4*	7.3	1.4*	12.0	2.2*
Waskish Peat HA + TCDD	21.3	2.5*	19.7	5.0	48.8	4.4*	43.0	16.3	87.6	33.5	112.1	25.9
Suwannee River FA	0.3	1.0	1.9	0.3*	0.3	0.3	1.8	0.4	0.8	0.9	5.0	2.7*
Suwannee River FA + TCDD	21.4	1.7*	17.2	4.0	47.5	0.7*	36.7	13.1	91.5	12.3	85.9	13.4
Nordic Aquatic FA	0.7	1.1	3.0	5.3	1.7	2.3	3.2	1.4	1.9	1.0	6.0	1.3*
Nordic Aquatic FA + TCDD	21.1	3.2*	17.3	4.2	45.9	3.5*	35.5	10.5	103.1	11.8	107.5	10.8
Suwannee River NOM	2.2	0.9	1.5	1.5	1.6	0.8	2.4	1.6	5.6	1.5*	10.5	1.8*
Suwannee River NOM + TCDD	22.5	2.6*	18.3	3.7	48.5	7.1	40.3	12.7	102.1	10.9	104.1	2.4
Nordic Reservoir NOM	0.2	1.6	0.6	2.1	1.6	0.2	2.2	1.8	0.6	0.5	6.6	0.8*
Nordic Reservoir NOM + TCDD	20.3	1.1*	14.5	2.4	47.7	7.2	39.9	4.9	88.3	24.9	102.1	21.2

* Denotes values different ($p < 0.05$) from either blank value (for pure HS – the first row from each couple), or TCDD value (for HS + TCDD values – the second row from each couple). Values represent the mean \pm SD of six independent experiments (for TCDD ~ 1.2 pM, HS ~ 17 mg L⁻¹) or three independent experiments (other HS–TCDD combinations), each performed in triplicates.

time POPs can interact with HS and result different toxicities. This ageing of HS–TCDD mixtures simulated exposure of H4IIE-*luc* to HS–TCDD mixtures preincubated for various periods of time.

Bioassay-derived data were examined statistically using Statistica for Windows 8.0 (StatSoft, USA). Comparison of AhR-mediated activity of HS samples with blank value, and [HS–TCDD] samples with TCDD alone was performed using unpaired *t*-test. Level of significance was chosen $p = 0.05$.

3. Results

Of the 12 HS tested, six (only HA, no FA or NOM samples) elicited a significant ($p < 0.05$) AhR-mediated activity in H4IIE-*luc* cells at all tested concentrations (Table 1, and Fig. 1 – the first column from each couple). None of the six AhR-active HA were able to completely activate AhR even in extreme concentrations ($\sim 300 \text{ mg L}^{-1}$); their greatest AhR-activation reached 40–80% of TCDD_{max}-activation (not depicted). As documented in our previous

paper (Janosek et al., 2007), observed AhR-mediated activity of HA is not caused by persistent pollutants such as PCDD (polychlorinated dibenzo-*p*-dioxins), PCDF (polychlorinated dibenzofurans) or PCB (polychlorinated biphenyls), but entirely by the non-persistent fraction of HA. This fraction could potentially contain some non-persistent AhR-active pollutants, such as PAH (polycyclic aromatic hydrocarbons). Nevertheless, TEQ calculated from chemical data for 16 U.S.EPA priority PAH (indication of possible anthropogenic contamination) was less than 1% of bioassay-derived TEQ for the HA (Janosek et al., 2007).

In the simultaneous exposure to environmentally relevant concentration of HS (17 mg L^{-1}) together with the lesser TCDD concentration (1.2 pM), statistically significant ($p < 0.05$) effects were observed. In the HS–TCDD mixtures at TCDD concentrations of 1.2 and 3.7 pM, enhancement of AhR-dependent activation by HS was observed (Fig. 1, the second column from each couple, and Table 1). Additive effects were observed in the case of AhR-active HS in the presence of TCDD (Fig. 1; first six couples). Enhancement

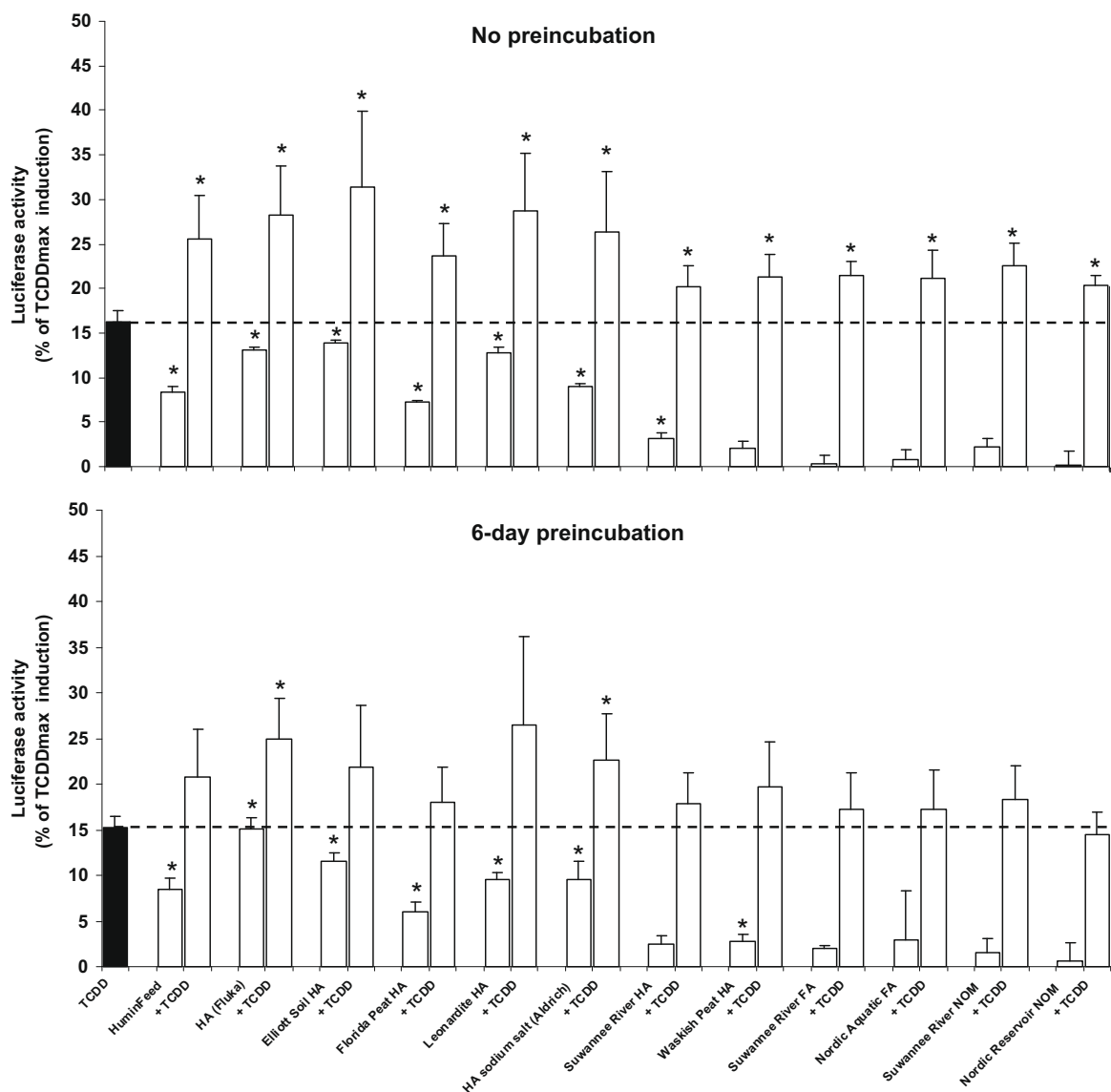


Fig. 1. AhR-mediated activity of 12 samples of HS determined in H4IIE-*luc* cells; black column and the dashed line at the level of 15% correspond to the response of 1.2 pM TCDD; the first column from each couple to 17 mg L^{-1} HS; the second column from each couple to co-exposure HS and TCDD. * denotes values different ($p < 0.05$) from either blank value (for pure HS – the first column from each couple), or TCDD value (for HS + TCDD values – the second column from each couple). Values represent the mean \pm SD of six independent experiments, each performed in triplicates.

was observed in the case of AhR-non active HS (especially FA and NOM) in the presence of TCDD. In these cases, AhR-mediated activities caused by pure FA or NOM were not significantly different from that of the blank, but FA/NOM–TCDD mixtures caused significantly greater AhR-mediated transactivation than TCDD alone (Fig. 1).

In the HS–TCDD mixtures with 500 pM TCDD (AhR-saturation level), no significant changes were observed except for the Florida Peat HA–TCDD mixture where additional AhR-activation was observed (Table 1). Nevertheless, no significant change at this excessive concentrations was expected because of the saturation of AhR by excessive amount of strong ligand (TCDD at 500 pM).

Preincubation for 1, 4, or 24 h caused no statistically significant differences compared to the corresponding non-preincubated samples (data not presented). Significant differences were observed only between samples without preincubation and samples preincubated for 6 days (Fig. 1 and Table 1). At a TCDD concentration of 1.2 pM (HS \sim 17 mg L⁻¹), the AhR-mediated effect of HS–TCDD mixture that had been preincubated for 6 days was on average 18% less than that of the analogous but non-preincubated samples ($p < 0.05$) at a TCDD concentration of 3.7 pM (HS \sim 50 mg L⁻¹), the AhR-mediated effect of HS–TCDD mixture preincubated for 6 days was on average 13% less than the analogous but non-preincubated samples ($p < 0.05$). At a TCDD concentration of 500 pM (HS \sim 150 mg L⁻¹), there was no significant change between the average AhR-mediated effect of preincubated and non-preincubated HS–TCDD mixtures. This is not surprising considering that the used concentration of TCDD (500 pM) was fully saturating the AhR. However, these concentrations of both TCDD and HS were excessive and not environmentally relevant.

4. Discussion

According to the “receptor-ligand interaction theory” the *partial agonists* (probably AhR-active HS) of appropriate receptor (AhR) might suppress the ability of *full agonist* (TCDD) to activate this receptor (Zhu, 2005). Thus, AhR-active HS could bind rather weakly to the AhR, but block binding of possible dioxin-like pollutant to AhR, which would hypothetically protect organism against consequent adverse physiological effects. Nevertheless, our results with binary mixtures of both HS and TCDD (Fig. 1 and Table 1) do not support this theory. We have observed either enhancement, addition or no significant change of TCDD-caused AhR-mediated effect by both AhR-active and inactive HS, but no significant decrease of AhR-mediated effect at any concentration without preincubation.

Enhancement of AhR-mediated effects of TCDD by dissolved HS, especially after longer-term preincubation (Fig. 1 and Table 1), generally at lesser TCDD concentration, can be explained by combination of at least three factors – *addition*, *facilitation* and *sorption*. *Addition* means simple addition of AhR-mediated effects of both TCDD and AhR-active HS samples. *Facilitation* means an increase of bioavailability of TCDD (highly hydrophobic compound) by both enhancement of its water solubility and changes in biomembrane permeability by dissolved organic matter (DOM). Such enhancement of water solubility was observed for similar hydrophobic compounds benzo[a]pyrene and chlorobiphenyls in mixture with dissolved organic matter (Doring and Marschner, 1998), while changes in biomembrane permeability were observed as a result of HA-biomembrane binding (Glover and Wood, 2005). *Sorption* is a well known ability of hydrophobic compounds to bind onto HS structures (Servos et al., 1992). TCDD exerts sorption partition coefficient ($\log K_{OC}$) of 6.1 that implicates sorptive interaction of

TCDD with HS that can have various ecotoxicological consequences. The sorption could lead to the decrease of TCDD bioavailability and thus ecotoxicity, but other HS properties (e.g. above mentioned facilitative properties) can mitigate this decrease of ecotoxicity.

In summary, AhR-mediated effects are the result of combined action of all the mentioned mechanisms. Additive and facilitative effects can be seen especially for lower concentrations of TCDD–HS mixtures without preincubation, while sorption of TCDD on HS, during the longer preincubation can lead to the decrease in AhR-mediated activity of HS–TCDD mixtures at lesser concentrations. Nevertheless, to generalize our results that HS can add to or alter the toxicity of POPs, it is necessary to assess interaction of HS with more AhR-active pollutants, such as e.g. AhR-active polychlorinated biphenyls or polycyclic aromatic hydrocarbons. Moreover, to generalize these findings to *in vivo* level, it would be necessary to carry out analogous experiments using living organisms. There are some recent studies with fish describing additive effect of coal-derived HA and crude oil on expression of cytochrome P450 1A (Matsuo et al., 2006) that is directly linked to AhR-activation. These findings indicate that observed *in vitro* results could be also observed if tested *in vivo*, even though the potential influence of biotransformation in whole organism which might lead to the disruption of the complex between HS and lipophilic xenobiotics can alter the internal exposure.

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