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***In vitro* profiling of the potential endocrine disrupting activities affecting steroid and aryl hydrocarbon receptors of compounds and mixtures prevalent in human drinking water resources**

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Abstract

Prioritizing chemicals posing threats to drinking water resources is crucial for legislation considering the cost of water treatment, remediation, and monitoring. We profiled *in vitro* potential endocrine disrupting activities (both agonistic and antagonistic) of 18 contaminants most prevalent in Walloon raw water resources intended for drinking water production, including several compound groups: pesticides, perfluorinated compounds, polycyclic aromatic hydrocarbons, a corrosion inhibitor, and bisphenol A. Mixtures thereof relevant for human realistic exposure were also investigated. Seven luciferase reporter gene cell lines were used *i.e.* three (human and rat) responsive to dioxins through the aryl hydrocarbon

receptor (AhR) and four (human) responsive to steroids through the estrogen (ER), androgen (AR), progesterone (PR), and glucocorticoid (GR) receptors. Among the 18 compounds, ten caused at least one response in at least one receptor. Specifically, chlorpyrifos, bisphenol A, fluoranthene, phenanthrene, and benzo[a]pyrene displayed significant activities on several receptors. Bisphenol A agonized ER, but abolished the cells' response to androgen and progesterone. While fluoranthene and phenanthrene strongly reduced human AhR and AR transactivation, benzo[a]pyrene strongly activated AhR and ER, but inhibited GR and AR. In human breast cancer cells, benzo[a]pyrene dramatically activated AhR, inducing a 10-fold higher response than 2,3,7,8-tetrachlorodibenzodioxin (TCDD) at concentrations possibly found realistically in human blood. The mixture of the 18 compounds exerted both ER and rat AhR agonism, with the main contribution being from benzo[a]pyrene or its combination with bisphenol A. Moreover, the mixture significantly inhibited TCDD-induced CYP1A activity (detected only by EROD assays) in human hepatoma cells.

Keywords: Raw water; Reporter gene assays; Endocrine disrupting activities; Aryl hydrocarbon receptor; Steroid receptors; Benzo[a]pyrene.

Highlights:

- Endocrine disrupting *in vitro* profile of 18 pollutants in drinking water resources.
- Chlorpyrifos, BPA, fluoranthene, phenanthrene, B[a]P display significant activities.
- B[a]P strongly induces AhR in human breast cancer cells to 10-fold higher than TCDD.
- The mixture exerts ER and rat AhR agonisms due to B[a]P or its combination with BPA.
- EROD assays can detect effects of the mixture on TCDD-induced CYP1A activity in human hepatoma.

1. Introduction

Since drinking water is an unavoidable route exposing humans to pollutants, access to safe drinking water is crucial for public health¹. Several legislations impose maximal limits of contaminants for the quality of water intended for human consumption, e.g. the Safe Drinking Water Act (SDWA) in the US², the Drinking Water Guidelines (ADWG) in Australia³, and the Drinking Water Directive 98/83/EC in the EU⁴. Endocrine disruptors (EDs), a group of exogenous substances that can interfere with the endocrine system thereby affecting the health of animals and humans, are of particular concern. Exposure to EDs even at low doses is associated with several health problems including abnormal reproductive activity, high incidence of cancers, and metabolic disorders e.g. obesity and cardiovascular diseases⁵⁻⁷.

EDs can alter the endocrine system in various ways, one of them is by affecting the activities of the crucial transcriptional factors e.g. the steroid (estrogen (ER), androgen (AR), glucocorticoid (GR), and progesterone (PR)) receptors and the aryl hydrocarbon receptor (AhR) which controls xenobiotic metabolism^{7,8}. The steroid receptors are regulated by steroids e.g. 17 β -estradiol (E2), 5- α -dihydrotestosterone (DHT), dexamethasone (Dex), progesterone (P4), respectively, while 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the typical ligand of the AhR. The ligand-bound receptors bind to their cognate DNA sequences (*i.e.* steroid or dioxin responsive elements, respectively) located in the promotor regions of their target genes to activate gene transcription^{9,10}.

Luciferase reporter gene assays take advantage of this series of events to establish reporter cell lines that are able to respond to the presence of ligands/stimuli¹¹. They thus accurately reproduce the mechanism of action of these compounds at the cellular level, as in a living organism¹². They are also able to discriminate between agonistic and antagonistic activities (by co-exposing the cells to the test compound and a typical agonist)¹¹. Moreover, they have been internationally validated to provide mechanistic and semi-quantitative data^{13,14} and have

shown a good correlation with *in vivo* experiments for several EDs^{15,16}. They are also able to reveal unknown properties especially of complex mixtures¹¹.

While there is still a need for research on protecting human health against the effects of EDs in drinking water⁷, especially in mixture forms which are more relevant for human exposure, prioritizing chemicals posing the threats is crucial considering the cost of water treatment, remediation, and monitoring. The Walloon Government in Belgium has recently funded the BIODIEN project (“search for EDs in waters”) aiming to quantify the contamination by specific EDs of several Walloon and Brussels water bodies. Among the 194 compounds monitored, 58 have been quantified at least once in 100 samples of raw water (surface and ground) intended for drinking water production¹⁷. However, the potential health effects of these chemicals as individuals and as mixtures have not yet been investigated, making difficult to prioritize the regulation of certain compounds/groups.

In this study, the endocrine disrupting activities of 18 compounds most frequently found in Wallonia raw water intended for drinking water production and their mixtures were investigated using luciferase reporter gene assays for five receptors (AhR, ER, AR, GR, and PR). The aims were to (a) assess both transcriptional agonistic and antagonistic activities of these compounds individually and in mixtures towards the five receptors, (b) identify the most concerning endocrine disrupting compound(s), and (c) identify the main compound(s) responsible for the observed effects of the mixtures.

2. Materials and methods

2.1. Chemicals and suppliers

We selected 19 compounds based on their highest frequency of quantification (> 8%) in the raw water samples from the BIODIEN project. VIS01 was excluded since no analytical standard available on the market. The main results of their monitoring during the BIODIEN

project and their suppliers are given in Table 1. The 18 selected compounds belong to several groups: eight pesticides and pesticide metabolites, five perfluorinated compounds (PFCs), three polycyclic aromatic hydrocarbons (PAHs), a corrosion inhibitor (1H-benzotriazole), and a plasticizer (bisphenol A, BPA). Reference standards, E2, DHT, Dex, and P4 were supplied by Sigma Aldrich (Missouri, USA), while TCDD was provided by Wellington (Campro Scientific, The Netherlands). All chemicals were dissolved in dimethylsulfoxide (DMSO, Acros Organics, France) and stored at -20°C . Tested concentration ranged from $1.25\ \mu\text{M}$ to $40\ \mu\text{M}$ in a series of six 2-fold dilutions, which has been shown to be suitable for the reporter gene assays without cytotoxicity^{18,19}.

2.2. Mixture preparation

A mixture of the 18 chemicals called the ED mixture, was constructed according to the maximum detected concentrations (C_{max}) in the raw water samples by pooling each component from their respective stock solutions. The concentrations of pesticides and PAHs in the mixture were measured by liquid chromatography- tandem mass spectrometry (LC-MS/MS) and gas chromatography-tandem mass spectrometry (GC-MS/MS) using in-house methods available in Société Wallonne des Eaux (SWDE) (see "measured concentration- C_m " in Table 1). PAHs were present in the mixture at lower concentration than expected, which could be due to their absorption by the glass container. To study the possible interactions among compound groups, a PFC mixture (five PFCs), a PEST mixture (eight pesticides, 1H-benzotriazole and BPA), and a PAH mixture (three PAHs) were also prepared. The tested concentrations of the mixtures are given as "fold maximal water levels" relative to their concentrations present in water samples (Table 1). The maximum final tested concentration (C_t) was 1000 fold, except desphenyl-chloridazon (DPC) which was 100 fold due to its very high concentration in water (it was not possible to make up 1000 fold, which corresponds to $148\ 10^{-6}\ \text{M}$, due to stock unavailability).

2.3. Reporter gene cell lines

Seven genetically modified cell lines expressing the reporter gene luciferase were used. Three dioxin responsive cell lines: rat hepatoma DR-H4IIE from BioDetection System (Amsterdam, The Netherlands), human hepatoma HepG2-Luc and human mammary gland T47-D-Luc (both produced on site in Liege, Belgium)²⁰, were used to compare the AhR transcriptional activity between species (rat and human) and tissues (liver and mammary gland). DR-H4IIE cells were stably transformed with a vector containing four native dioxin responsive elements (DREs) from the upstream promotor of the mouse *Cyp1a1* gene with a MMTV (mouse mammary tumor virus) promoter²¹, while four synthetic DREs regulating the thymidine kinase promoter were in both T47-D-Luc and HepG2-Luc cells²⁰. The four human steroid responsive cell lines (ER-MMV-Luc, TARM-Luc, PR-TM-Luc and TGRM-Luc) addressing respectively the estrogen, androgen, progesterone and glucocorticoid receptor, were previously described¹¹. ER-MMV-Luc cells were MCF-7 cells transformed with a vector containing the luciferase gene controlled by a vitellogenin promoter. The other three steroid reporter cells were derived from T47-D cells sharing a common vector containing the MMTV-LTR (long terminal repeat) promoter, inducible by glucocorticoid, progesterone, and androgen ligands¹¹. While ER-MMV-Luc and PR-TM-Luc responded through their endogenous receptors, TGRM-Luc and TARM-Luc were stably transformed with an additional expression vector coding for, respectively human GR and AR.

The cells were maintained in MEM α (ThermoFisher Scientific, Massachusetts, USA), except for ER-MMV-Luc in DMEM (ThermoFisher Scientific) supplemented with 1 μ g/mL bovine pancreas insulin (Sigma Aldrich). Both media were supplemented with 10% v/v fetal bovine serum (FBS) (Greiner, Kremsmünster, Austria), 50 IU/mL penicillin and 50 μ g/mL streptomycin (Sigma Aldrich). The cells were incubated in a H₂O saturated atmosphere containing 5% CO₂, at 37°C. In the rest of the manuscript, MEM α and DMEM will stand for the respective medium supplemented with FBS and penicillin and streptomycin.

2.4. Reporter gene assays

Different protocols for each cell line were described in detail elsewhere^{11,20}. Briefly, after 24h of cell seeding in white clear-bottomed 96 well microplates (Greiner) with 90% cell confluence, the medium was refreshed and the cells were exposed to a dilution series of the tested compound/mixture mentioned above. A reference curve of the reference ligand along with the blank DMSO was performed on each plate for quality control. For co-exposure tests, the same dilution series was used on cells that were cotreated with a constant concentration of the reference compound, close to its EC₅₀ (Table S1). The AhR reporter cells were exposed for 24h in MEM α , while the steroid reporter cells were exposed for 48h in DMEM without phenol red and supplemented with charcoal stripped FBS to eliminate natural steroid activators. After exposure, the cells were lysed by solutions containing Triton X100 (Sigma Aldrich). The luminescence signal was recorded by adding luciferin (Promega, Wisconsin, USA) and ATP (Roche Diagnostics, Rotkreuz, Switzerland) to the cell lysate and measured by a luminometer (ORION II, Berthold Detection System, Pforzheim, Germany). The final concentration of DMSO was 0.2% and 0.3% for the agonistic and co-exposure tests, respectively. All tests were performed in at least three independent experiments, each in triplicate. The cytotoxicity was examined by visually inspecting the cell morphology and density under the microscope before cell lysis.

Table S1 shows the characteristics of the seven reporter gene cell lines. In general, the cell lines performed similarly as in the previous studies *i.e.* similar EC₅₀ and fold induction^{11,19,22}, except a reduction of the maximal fold induction seen for TGRM-Luc (from around 2000 fold to 320 fold) and an increase of EC₅₀ for PR-TM-Luc ($705 \pm 40 \times 10^{-9}$ M compared to 1.5×10^{-9} M), however the latter value was close to that observed of $330 \pm 0.9 \times 10^{-9}$ M in a recent study²³.

2.5. EROD assays in HepG2-Luc and T47-D-Luc

EROD assays measure the activity of ethoxyresorufin-O-deethylase (eroD) enzyme that is associated with CYP1A converting ethoxyresorufin to fluorescent resorufin. They were thus used to determine the CYP1A activity of the mixtures in HepG2-Luc and T47-D-Luc. The EROD assays were performed according to Schiwy *et al.*, (2015)²⁴ with the exposure conditions as described for the reporter gene assays. However, to adjust to the much higher TCDD sensitivity of EROD assays towards the AhR from both human cell lines, the maximum TCDD concentrations were 20 and 40-fold lower (500 pM for HepG2-Luc and 250 pM for T47-D-Luc) than in the reporter gene assays (10 nM for both), and the corresponding TCDD concentration used in the co-exposure tests was respectively 100 pM and 50 pM, instead of 650 pM and 150 pM. After 24h exposure, 100 µl of working solution, freshly prepared by dissolving 7-ethoxyresorufin (5 µM) (Sigma Aldrich) in DMEM without phenol red, was added into 50 µl lysed-cell extract, followed by an incubation for 30 minutes at 37°C in the dark. To terminate the reaction, 100 µl chilled to 4°C methanol (Merck, Massachusetts, USA) was added. The fluorescence signal of resorufin was measured at 550/595 nm using a Perkin Elmer VICTOR X5 MultiLabel Plate Reader, Germany.

2.6. Calculations of relative responses RR, EC₅₀, IC₅₀, and efficacy

The cells' responses were obtained as relative light units (RLUs). The activities of the compound/mixture (n) for both reporter gene and EROD assays were presented as relative responses (RR_{a(n)} and RR_{i(n)} (%) for agonistic and co-exposure tests, respectively) and determined as:

$$(1) RR_{a(n)} = \frac{R_{a(n)} - R_{DMSO}}{R_{(ref-max)} - R_{DMSO}} \times 100$$

$$(2) RR_{i(n)} = \frac{R_{i(n)} - R_{DMSO}}{R_{(ref-EC50)} - R_{DMSO}} \times 100$$

RR_{a(n)} (Eq. 1) or RR_{i(n)} (Eq. 2) was the percentage (%) of the agonistic (R_{a(n)}) or co-exposure (R_{i(n)}) response of the compound/mixture (n) compared to the maximum response (R_(ref-max)) or

to the response of the spike-in EC₅₀ (R_(ref-EC50)) of the reference compound, both after subtraction of the vehicle (DMSO) response (R_{DMSO}). A value of RR_{i(n)} (%) higher than 100% reflected an additive/enhancing action of the compound/mixture on top of the effect caused by the presence of the reference compound at EC₅₀ (marked as “+” in Table 3). Meanwhile, a value less than 100% demonstrated the antagonistic potential of the compound/mixture to inhibit the activity of the reference compound at EC₅₀, thus, the lower the RR_{i(n)} (%) was, the stronger the antagonistic activity.

The dose-response curves were generated by fitting a four-parameter non-linear stimulation or inhibition regression (Graphpad PRISM software, version 7, San Diego, California, USA) for agonistic (Eq. 3) or antagonistic (Eq. 4) tests:

$$(3) RR_{a(n)x} = B + \frac{x^H(T - B)}{x^H + EC_{50}^H}$$

$$(4) RR_{i(n)x} = B + \frac{T - B}{1 + \frac{x^H}{IC_{50}^H}}$$

where x was the concentration of the tested compound/mixture inducing the relative response RR_{a(n)x} or RR_{i(n)x}. EC₅₀ and IC₅₀ were the half maximal effective concentrations for an agonist and antagonist, respectively²⁵, note that no IC₅₀ was determined for the additive activities. B = bottom, T = Top, H = Hill slope. B was constrained higher than 0, respectively for the minimum and maximum responses in agonistic and antagonistic tests. A compound/mixture was considered active if its effect was significantly different from that of DMSO alone (ANOVA, Graphpad PRISM, p < 0.05). The efficacy (RR_{a/max} or RR_{i/max} (%)) for agonistic or antagonistic activities, respectively) was determined as the significant maximum effect of the compound/mixture (ANOVA, Graphpad PRISM, p < 0.05). When no full dose response curve was obtained, RR_{a/max} or RR_{i/max} was assigned as the effect induced by the highest tested concentration of the compound/mixture.

3. Results

3.1. Activities of the 18 compounds

No cytotoxicity was recorded for any of the 18 compounds within the concentration range tested for both agonistic and co-exposure experiments. Among eight pesticides, methyl-desphenyl-chloridazon (Me-DPC) and desethylatrazine exerted weak agonistic responses that were best observed when cells were cotreated with TCDD, respectively in DR-H4IIE and T47-D-Luc (Figures 1B and 1F'), while atrazine antagonized TCDD activity in HepG2-Luc with $RR_{i/max} = 70\%$ (Figure 1D). The most active pesticide was chlorpyrifos, inducing in DR-H4IIE and ER-MMV-Luc both weak agonistic ($RR_{a/max} = \sim 10\%$) (Figures 1A and 1G) and significant antagonistic responses (Figure 1B and 1H) ($RR_{i/max} = 62\%$ and 43% , respectively), while it was only agonistic ($RR_{a/max} = 27\%$) in T47-D-Luc (Figure 1E') and only antagonistic ($RR_{i/max} = 68\%$) in PR-TM-Luc (Figure 1J).

BPA strongly induced the response of ER-MMV-Luc with $RR_{a/max} = 119\%$, higher than the maximum response of E2 (Figure 1G), but did not cause any effect when cells were co-exposed with E2. It also inhibited the responses of the four T47D-derived transgenic lines in the co-exposure tests, moderately in T47-D-Luc and TGRM-Luc (Figures 1F' and 1I), and particularly strongly in PR-TM-Luc and TARM-Luc with $RR_{i/max} = 15\%$ (Figures 1J and 1K).

The most potent group was PAHs, which caused a response in all seven cell lines, except phenanthrene which displayed no response in ER-MMV-Luc, TGRM-Luc, and PR-TM-Luc. Interestingly, fluoranthene and phenanthrene behaved differently from benzo[a]pyrene (B[a]P). While phenanthrene did not induce agonistic activities in any cell line, fluoranthene caused a weak agonistic response, with $RR_{a/max} = 21\%$ and 28% in DR-H4IIE and ER-MMV-Luc, respectively (Table 2). However, they both strongly inhibited the TCDD response with $RR_{i/max} = \sim 40\%$ in DR-H4IIE, and even more strongly in HepG2-Luc and T47-D-Luc ($RR_{i/max} = \sim 10\%$). Fluoranthene also antagonized P4 in PR-TM-Luc and DHT in TARM-Luc with $RR_{i/max}$

= 62% and 16%, respectively, whereas phenanthrene inhibited only the TARM-Luc response with $RR_{i/max} = 34\%$ (Table 3).

B[a]P was a potent agonist in DR-H4IIE with $RR_{a/max} = 171\%$, nearly twice the cells' maximum response to TCDD (Figure 1A). The effect remained when it was co-exposed with EC_{50} TCDD with $RR_{i/max} = 280\%$ (Figure 1B), since this resulted in a final $RR_{a(C19)/max} = 126\%$ after the spike-in EC_{50} reference was subtracted and the response $RR_{a(C19)}$ was compared to the maximum response of the reference compound. B[a]P was also able to agonize human AhR in hepatoma HepG2-Luc, but to a lesser extent ($RR_{a/max} = 27\%$) (Figure 1C), causing a partially antagonistic activity in this cell line when co-exposing with TCDD, showing an antagonistic activity up to 10 μ M and an agonistic activity at higher concentrations (Figure 1D). Surprisingly, B[a]P induced an extremely strong agonistic response in human mammary gland carcinoma T47-D-Luc, with $RR_{a/max} = 1061\%$ (Figure 1E), which remained similar in the co-exposure test with $RR_{i/max} = 2713\%$ or a final $RR_{a(C19)/max} = 1266\%$ (Figure 1F), indicating that the presence of TCDD did not affect the B[a]P activity in this cell line.

In ER-MMV-Luc, B[a]P induced a strong agonistic response of $RR_{a/max} = 253\%$, while in the presence of E2, an inhibition was observed at lower concentrations and an activation at higher concentration, parallel to the agonistic curve with $RR_{i/max} = 233\%$ or $RR_{a(C19)/max} = 102\%$, less than half of $RR_{a/max}$ (Figures 1G and 1H). It also caused a similar response in PR-TM-Luc, but only when co-exposing the cells with P4, with $RR_{i/max} = 172\%$ or $RR_{a(C19)/max} = 111\%$ (Figure 1J). Moreover, B[a]P significantly antagonized both Dex and DHT in TGRM-Luc and TARM-Luc with $RR_{i/max} = 36\%$ and 9%, respectively. All these data are summarized in Tables 2 and 3 and Figure 1S.

3.2. Activities of the mixtures

No activity was observed for the PFC mixture. In DR-H4IIE, the ED mixture showed an agonistic activity similar to that of the PAH mixture with $RR_{a/max} = 42\%$ and 45%, respectively,

while the PEST mixture activation was lower ($RR_{a/\max} = 17\%$). In comparison, the ER-MMV-Luc responded similarly to both the PAH and PEST mixtures with $RR_{a/\max} = 17\%$ and 16% , respectively, while the ED mixture caused a strong agonistic effect ($RR_{a/\max} = 32\%$) (Table 2).

Additive responses of these mixtures were observed when the cells were co-exposed with TCDD or E2 (Table 3). However, the additive responses were not significant in DR-H4IIE with only $RR_{i/\max} = \sim 125\%$ for both ED and PAH mixtures, and only 115% for the PEST mixture. A similar result was obtained in ER-MMV-Luc ($RR_{i/\max}$ PAH and ED = 145% and PEST = 122%). Since full dose response curves were not obtained within the tested concentration range, no values could be deduced for EC_{50} and for the additive IC_{50} (Tables 2 and 3).

From Table 2, it seems that the observed agonistic effects of the PAH and ED mixtures in DR-H4IIE were due to the agonistic activity of B[a]P, while the agonistic effect seen for the ED mixture in ER-MMV-Luc was due to the combination of B[a]P and BPA. This assumption was confirmed by testing the concentrations of B[a]P, BPA, and B[a]P + BPA corresponding to their concentrations in the mixtures (Figure 2A). No major contributor could be identified for the weak agonism of the PEST mixture in DR-H4IIE.

3.3. EROD assays with the mixtures in HepG2-Luc and T47-D-Luc

The CYP1A inducing activity of the mixtures on the two human AhR reporter cell lines was determined using the EROD assays. In T47-D-Luc, while only an agonistic effect was observed for the ED mixture in the reporter gene assays, there was a moderate agonistic response seen for the PAH mixture with $RR_{a/\max} = 45\%$ (Figure 2B1) and weak antagonistic responses seen for the PEST and ED mixtures ($RR_{i/\max} < 80\%$) (data not shown). The PEST and ED mixtures induced the same antagonistic responses in HepG2-Luc with IC_{50} PEST = 180 ± 25 and IC_{50} ED = 177 ± 23 fold and $RR_{i/\max}$ PEST = 16% and $RR_{i/\max}$ ED = 14% , while the PAH mixture displayed a weaker response of $RR_{i/\max} = 67\%$ (Figure 2B2).

4. Discussion

4.1. Endocrine disrupting activities of the 18 compounds

This study is one of the few studies^{22,23,26–30} using a panel of multiple cell-based bioassays (seven reporter gene cell lines including five receptors (AhR, ER, GR, PR, and AR)) for *in vitro* profiling of the potential endocrine disrupting activity of multiple chemicals which were frequently detected in Wallonia raw water intended for drinking water production. Appearing with relatively high frequency of quantification (FOQ), several herbicides (DPC, metolachlor ESA, BH479-8, simazine), 1H-benzotriazole (FOQ >~40%), and the five PFCs (FOQ = ~20%) did not display a significant response in these bioassays, except a weak response ($RR_{a/max} < 10\%$) for PFOS and PFHxS in ER-MMV-Luc. Hence, they are unlikely to induce any endocrine disrupting activity via these receptors, although they can induce health risks in humans by several other molecular mechanisms e.g. PFCs^{28,31} or metolachlor³⁰. Three compounds (Me-DPC, desethylatrazine and atrazine) triggered an AhR response in a species- and cell type-specific manner. To our best knowledge, these results are the first recorded for these cell lines. The compounds were frequently detected in raw water samples; hence their activities may be a matter of concern. Meanwhile, chlorpyrifos, BPA, fluoranthene, phenanthrene and B[a]P were active on several receptors. AhR and ER were more general targets for both agonistic and antagonistic activities, responding to respectively eight and six compounds, while GR, PR and AR were targets only for antagonism (see Figure S1 for detailed clusters).

Chlorpyrifos caused both agonistic and antagonistic activities towards AhR and ER and only antagonized PR. Therefore, chlorpyrifos appears to be a potential ED. The result was consistent with previous *in vitro* reports concerning this compound showing activation of AhR^{30,32} and ER^{33–35} and with *in vivo* estrogenic activity observed in mammary gland in female rats³⁶. The EU legislation imposes a limit of 0.1 µg/L in drinking water for total pesticides⁴, which is equivalent to 0.3 nM chlorpyrifos, 10000 fold lower than the lowest observed effect concentration of chlorpyrifos at 5 µM (Figures 1B, E', H, and J) in our study. However, due to

a high bioconcentration factor (e.g. 100 to 5100 in fish³⁷), a high documented toxicological activity^{35,36,38} including this study, a considerably high contamination potential due to the current use in the US and a very recent ban in the EU, the monitoring of chlorpyrifos should be specifically indicated in the legislation. In US, potential exposure to chlorpyrifos in drinking water based on model estimates continues to exceed safe levels³⁹.

BPA is a ubiquitous contaminant, prevalent also in water, including drinking water^{40–42}, whose ability to activate ER-mediated pathways is well documented^{43–45} and has been confirmed in this study. However, its activity does not always involve estrogen-mediated mechanisms as shown in human endometrial stromal fibroblasts⁴⁶ or in human endometrial adenocarcinoma cells where it prevented the proteasome-mediated degradation of ER β ⁴⁷. In addition, BPA inhibited the activities of all the reference compounds in the T47-D transgenic cell lines: T47-D-Luc, TGRM-Luc, TARM-Luc, and PR-TM-Luc, while only anti-androgenic was previously shown in *in vitro* reporter gene assays^{26,27,48}. BPA was present in Wallonia raw water at C_{max} = 31 ng/L but rarely detected (FOQ = 9.1%)¹⁷. Currently, BPA does not appear to pose a threat to these drinking water sources. However, due to its significant activity, BPA should be listed for monitoring in the Council Directive 98/83/EC⁴.

PAHs are a diverse class of toxicants, among which several are well known as genotoxic carcinogens⁴⁹. Also, they activate AhR^{50–52} as one of their major toxicity pathways and interfere with estrogenic signaling through direct or indirect estrogen-like effects^{53,54} and anti-estrogenic effects^{54,55}. Besides, it was shown in the EU⁵⁶ that these compounds can be present in drinking water at concentrations above the maximal limits defined in the Drinking Water Directive⁴. We not only confirmed the finding of weak, but significant induction of the rat AhR response to fluoranthene (but not for phenanthrene) in DR-H4IIE⁵², but also showed that both strongly antagonized TCDD-induction in the human HepG2-Luc and T47-D-Luc. We also newly discovered the strong antagonistic effects of both compounds towards androgens in TARM-Luc (Table 3). Neither fluoranthene nor phenanthrene are in the list of the EU legislation for

water intended for human consumption⁴. Regarding their significant endocrine disrupting activities, especially anti-AhR and anti-AR towards human-based cell lines and their presence in water sources, we propose to add fluoranthene and phenanthrene to the list of drinking water monitoring in the EU.

B[a]P is known as a potent endocrine and growth disruptor, particularly altering the AhR pathway and displaying both estrogenic and anti-estrogenic activity in several systems^{30,57,58}. In this study, B[a]P significantly modified the transcriptional activities of all five receptors in all seven cell lines. It was a strong AhR agonist in DR-H4IIE and caused both agonistic and partially antagonistic activities in HepG2-Luc, as documented previously^{50,51}. B[a]P also displayed an agonistic and a partial antagonistic activity in ER-MMV-Luc. It was shown that the estrogenic activity of B[a]P in MCF-7 cells required the activation of the AhR and the subsequent induction of cytochrome P450 enzymes to produce estrogenic B[a]P metabolites, most notably 3-OH-B[a]P⁵⁹. Indeed, B[a]P failed to stimulate ER-dependent reporter luciferase activity in AhR-knockout MCF-7 cells, indicating that the estrogenic-activity of B[a]P is a “secondary” response driven by its activation of the AhR signaling pathway.

However, the most striking result was the enhanced response observed in T47-D-Luc exposed to B[a]P with a response 10-fold higher than the maximum response to TCDD. A “pure” AhR antagonist, GNF315⁶⁰ at 10 μ M inhibited the B[a]P-induced AhR activation in T47-D-Luc by more than half, from $RR_{a/max} = 1061\%$ to $RR_{a/max} = 346\%$ (Figure S2). However, the other AhR antagonist, CH-223191 did not affect the B[a]P activity. CH-223191 had also failed to inhibit the stimulation of AhR-DNA binding of beta-naphthoflavon (a PAH-like chemical)⁶¹. Meanwhile, ER antagonists (fadrozole and fulvestrant) at 1 μ M did not affect the cells' response to B[a]P (data not shown). Thus, the B[a]P-induced enhancement of AhR activation seems to be partially dependent on the AhR activity, but unrelated to the ER activity. This enhancement could be due to some mechanism acting downstream of AhR activation that is

triggered by B[a]P specifically in mammary gland cells. This is consistent with the observed enhancement (2.5-fold) of ER activation in ER-MMV-Luc, also derived from human mammary gland cells (MCF-7). T47-D cells are also used in the reporter cell-assays for progesterone, glucocorticoid and androgen. We also observed an enhanced response (about 2-fold) to progesterone in PR-TM-Luc, consistent with a specific enhancement mechanism in these cells, but antagonism against glucocorticoid and androgen. It is important to mention here that the parent T47-D cells produce their endogenous PR, which is thus also present in the TGRM-Luc and TARM-Luc that contain a transgene coding, respectively for exogenous GR or AR. This explains why the initial antagonism of GR and AR observed in these lines at lower B[a]P concentrations was dominated at higher concentrations by the enhanced PR response. Further investigations will be required to fully understand the actions of B[a]P in these cells. In addition, exposure to B(a)P has been shown to facilitate the migration and invasion of mammary epithelial cells (MCF-7 and MDA-MB-231) due to perturbation of the inflammation mediators and dysregulation of the tumorigenic miRNAs⁶².

Such an enhancement is extremely concerning in terms of human exposure to B[a]P which is mostly via food consumption rather than via drinking water. B[a]P exposure resulted in a maximal response (10-fold higher than TCDD) at only 10 μ M, while inductions similar to that of TCDD were already observed at 2.5 μ M in the human mammary gland T47-D-Luc. Although B[a]P is rapidly metabolized and thereby quickly eliminated from the body⁶³, B[a]P is still the ubiquitous PAH contaminant in human samples⁶⁴. B[a]P contamination can be found at 7.1 nM in the follicular fluid of women who smoke⁶⁵ or 20 nM in serum samples from autopsy cases of African American females⁶⁴. Therefore, a 100-fold increased exposure up to 2.5 μ M of B[a]P in blood could be realistic due to the chronic exposure of highly exposed sub-populations or acute exposure due to food contamination incidents.

4.2. Activities of the mixtures

The ED mixture consisted of the 18 compounds at their concentrations in a nM range, corresponding to 1000-fold their maximum water level, except for the two pesticides DPC and Me-DPC (Table 1). The ED, PAH and PEST mixtures caused an agonistic effect on AhR in DR-H4IIE and on ER in ER-MMV-Luc. The AhR agonistic effect of the ED and PAH mixtures could be reproduced using only their main contributor, B[a]P, while the very weak activity of the PEST mixtures was probably due to the addition of the activities of its components, each individually being too weak to be detected. In contrast, the ER agonistic activity could be attributed to the presence of either BPA (in PEST), B[a]P (in PAH), or both (in ED). Noticeably, the ED mixture already agonized rat AhR at concentration of 100 fold the water levels, (Figure 2A1) at which the mixture contained 14 nM B[a]P, a concentration realistically observed in human fluids^{64,65}.

None of the mixtures displayed any effect in either HepG2-Luc or T47-D-Luc in reporter gene assays, except for a weak agonistic effect of the ED mixture for both cell lines (Table 2). In contrast, EROD assays revealed a moderate agonistic effect for the PAH mixture in T47-D-Luc (Figure 2B1) and a strong antagonistic effect for the ED and PEST mixtures, and to a lesser extent, for the PAH mixture in HepG2-Luc (Figure 2B2). This greater sensitivity of EROD assays for measuring CYP1A activity, at least for the two human AhR cell lines, allowed for a better detection of agonistic, but also antagonistic activities of the mixtures, possibly due to the lower TCDD concentration used in co-exposure experiments. However, contrary to reporter gene assays, antagonism in EROD assays could also result from the inhibition of the CYP1A enzyme activity by the mixtures, which may not be detected in the reporter gene assays. The PEST mixture rather than the PAH mixture induced a similar inhibition on CYP1A activity in HepG2-Luc, indicating its components to be the actual chemicals responsible for this mixture effect. In fact, exposure to pesticides *i.e.* atrazine and chlorpyrifos showed an antagonistic effect on CYP enzymes in common carp gills⁶⁶, while benzotriazole induced down-regulation of CYP1A1 in the liver, gills and intestines of medakas⁶⁷ and 1-

aminobenzotriazole, along with other benzotriazole derivatives, is a well-known CYP inhibitor^{68,69}.

Conclusions

We tested the endocrine disrupting activities of 18 compounds shown to be present at trace concentrations in raw water intended for drinking water production in Wallonia. Both pure solutions of each compound and mixtures were studied for their ability to agonize and antagonize the transcriptional activities of a panel of five receptors, listed as AhR, ER, AR, PR and GR. Among these compounds, chlorpyrifos, BPA, fluoranthene, phenanthrene, and B[a]P demonstrated significant activities which involved more than one mechanism. Their presence in raw water source should be monitored more strictly. BPA, fluoranthene, and phenanthrene should be added to the list of monitoring due to their potential endocrine disrupting activities. B[a]P alone or with BPA were the main contributors for the agonistic activities of the ED and PAH mixtures towards the rat AhR the human ER. EROD assays were able to reveal the antagonistic effect of the mixtures in DR-HepG2 cells, which had not been observed in the reporter gene assays. The results revealed the multi-endocrine disrupting activities of several contaminants in raw water intended for drinking water production, some of them not being mentioned in the EU legislation relating to monitoring of drinking water quality. Their (antagonistic) effects on different receptors with the potential for additive or inhibitory effects in the mixtures should be considered in their risk assessments.

Conflict of interest: The authors declare that there is no conflict of interest.

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Figure 1. Dose-response curves of the active chemicals in (A-B) DR-H4IIE, (C-D) HepG2-Luc, (E-F') T47-D-Luc, (G-H) ER-MMV-Luc, (I) TGRM-Luc, (J) PR-TM-Luc, and (K) TARM-Luc, inducible respectively by aryl hydrocarbon, estrogen, glucocorticoid, progesterone, and androgen receptors. (Mean $\pm$ SD, $n = 3$, 0.2% or 0.3% DMSO for agonistic or co-exposure tests, respectively). Dash lines (100%) are the maximum response or the response of the corresponding reference compound in agonistic or co-exposure tests, respectively.

Figure 2. (A) Dose-response curves of the mixtures and BPA, B[a]P and BPA+B[a]P in the DR-H4IIE (A1) and ER-MMV-Luc (A2). (B) Dose-response curves of the mixtures in T47-D-Luc (B1) and HepG2-Luc (B2) in EROD assays. (Mean $\pm$ SD, $n = 3$, 0.2% or 0.3% DMSO for agonistic or co-exposure tests, respectively). Dash lines (100%) are the maximum response or the response of the corresponding reference compound in agonistic or co-exposure tests, respectively.

Table 1. List of the 19 compounds most frequently found in raw water samples in the Wallonia and Brussels areas¹⁷.

Compound	CAS	Supplier reference	FOQ (%)	C _{max} (ng/L)	C _m (ng/L)	C _t (μM)
Desphenyl-chloridazon (DPC)	6339-19-1	AKOS-006278170	82	21500	1230*	8.5*
Methyl-desphenyl-chloridazon (Me-DPC)	17254-80-7	DRE-C11322500	69.7	1930	1320	8.3
Desethylatrazine	6190-65-4	Sigma 36629	67.4	122	111	0.59
VIS 01/Chlorothalonil SA	1418095-02-9	Not included	55.1	772	ND	NA
Metolachlor ESA	171118-09-5	DRE-CA15171100	53.9	890	450	0.47
Atrazine	1912-24-9	Sigma 45330	44.2	101	101	1.3
Metazachlor ESA (BH479-8)	172960-62-2	DRE-CA14950020	40.4	186	130	0.4
1H-benzotriazole	95-14-7	Sigma 76457	34.7	186	ND	1.6
Perfluorooctanesulfonic acid (PFOS)	1763-23-1	Sigma 33829	29	19.95	ND	0.04
Perfluorooctanoic acid (PFOA)	335-67-1	Sigma 33824	28	5.51	ND	0.01
Perfluorohexanoic acid (PFHxA)	307-24-4	DRE-C15986910	23	11.53	ND	0.04
Perfluoroheptanoic acid (PFHpA)	375-85-9	Sigma 342041	18	2.18	ND	0.01
Perfluorohexanesulfonic acid (PFHxS)	355-46-4	Sigma 50929	13	15.02	ND	0.03
Simazine	122-34-9	Sigma 32059	10.5	23	28	0.14
Chlorpyrifos	2921-88-2	Sigma 45395	10.5	104	92	0.26
Bisphenol A (BPA)	80-05-7	Sigma 239658	9.1	31	ND	0.14
Fluoranthene	206-44-0	Sigma 45504	8.4	62	11	0.05
Phenanthrene	85-01-8	Sigma 695114	8.4	19	29	0.16
Benzo[a]pyrene (B[a]P)	50-32-8	Sigma B1760	8.4	70	29	0.12

FOQ: frequency of quantification (% of samples), C_{max}: maximum concentration quantified in raw water samples. C_m: measured concentration by LC-MS/MS and GC-MS/MS in the ED mixture. C_t: the maximum tested concentration in the ED mixture corresponding to 1000 fold maximum water levels, except 100 fold for DPC (*). ND: not determined.

Table 2. EC₅₀ and RR_{a/max} values of the agonistic responses to the selected 18 chemicals and four mixtures (n = 3, 0.2% DMSO).

Compound/mixture	DR-H4IIE		HepG2-Luc		T47-D-Luc		ER-MMV-Luc	
	EC ₅₀ ± SE	RR _{a/max}	EC ₅₀ ± SE	RR _{a/max}	EC ₅₀ ± SE	RR _{a/max}	EC ₅₀ ± SE	RR _{a/max}
DPC	-	-	-	-	-	-	-	-
Me-DPC	-	-	-	-	-	-	-	-
Desethylatrazine	-	-	-	-	24.1 ± 4.8	8	-	-
Metolachlor ESA	-	-	-	-	-	-	-	-
Atrazine	-	-	-	-	-	-	-	-
BH479-8	-	-	-	-	-	-	-	-
1H-benzotriazole	-	-	-	-	-	-	-	-
PFOS	-	-	-	-	-	-	ND	7
PFOA	-	-	-	-	-	-	-	-
PFHxA	-	-	-	-	-	-	-	-
PFHpA	-	-	-	-	-	-	-	-
PFHxS	-	-	-	-	-	-	ND	6
Simazine	-	-	-	-	-	-	-	-
Chlorpyrifos	10.5 ± 2.3	10	-	-	ND	27	22.6 ± 17.7	11
BPA	-	-	-	-	-	-	1.2 ± 0.1	119
Fluoranthene	ND	21	-	-	-	-	25.4 ± 6.4	28
Phenanthrene	-	-	-	-	-	-	-	-
B[a]P	ND	171	16.8 ± 2.3	27	4.2 ± 0.2	1061*	6.5 ± 0.4	253*
PFAA	-	-	-	-	-	-	-	-
PAH	ND	45	-	-	-	7	ND	17
PEST	ND	17	-	-	-	-	ND	16
ED	ND	42	ND	6	ND	11	ND	32

EC₅₀ (µM for individual compounds and fold water level for mixtures): the concentration inducing half of the maximum activation response; SE: Standard Error; RR_{a/max} (%): observed activation efficacy; ND: Not Determined. -: no response at concentration ≤ 40 µM. *Maximum efficacy observed within the tested concentrations.

Table 3. IC₅₀ and RR_{i/max} values of the co-exposure responses to the selected 18 chemicals and four mixtures (n = 3, 0.3% DMSO).

Compound/mixture	DR-H4IIE		Hep G2-Luc		T47-D-Luc		ER-MMV-Luc		TGRM-Luc		PR-TM-Luc		TARM-Luc	
	IC ₅₀ ± SE	RR _{i/max}	IC ₅₀ ± SE	RR _{i/max}	IC ₅₀ ± SE	RR _{i/max}	IC ₅₀ ± SE	RR _{i/max}	IC ₅₀ ± SE	RR _{i/max}	IC ₅₀ ± SE	RR _{i/max}	IC ₅₀ ± SE	RR _{i/max}
DPC	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Me-DPC	ND	125 ⁺	-	-	-	-	-	-	-	-	-	-	-	-
Desethylatrazine	-	-	-	-	ND	140 ⁺	-	-	-	-	-	-	-	-
Metolachlor ESA	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Atrazine	-	-	ND	70	-	-	-	-	-	-	-	-	-	-
BH479-8	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1H-benzotriazole	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PFOS	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PFOA	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PFHxA	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PFHpA	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PFHxS	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Simazine	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chlorpyrifos	24 ± 10	62	-	-	-	-	6.8 ± 5.2	43 [*]	-	-	44 ± 159.7	68 [*]	-	-
BPA	-	-	-	-	ND	71	-	-	5.8 ± 1.7	44	18.5 ± 6.3	15	4.3 ± 3	15 [*]
Fluoranthene	3 ± 0.3	47	11.6 ± 0.8	12	4.4 ± 0.3	11 [*]	-	-	-	-	13.9 ± 3.9	62 [*]	3.2 ± 2.6	16 [*]
Phenanthrene	15 ± 4	35	12.3 ± 1.2	12	7.9 ± 0.6	8 [*]	-	-	-	-	-	-	10.4 ± 2.5	34 [*]
B[a]P	ND	280 ⁺	ND	50 [*]	ND	2713 ⁺⁺	ND	233 ⁺⁺	2.3 ± 881	36 [*]	ND	172 ⁺⁺	2.3 ± 29	9 [*]
PFAA	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PAH	ND	124 ⁺	-	-	-	-	ND	145 ⁺	-	-	-	-	-	-
PEST	ND	115 ⁺	-	-	-	-	ND	122 ⁺	-	-	-	-	-	-
ED	ND	126 ⁺	-	-	-	-	ND	145 ⁺	-	-	-	-	-	-

IC₅₀ (µM for individual compounds and fold water level for mixtures): the concentration inducing half of the maximum inhibition response; SE: Standard Error; RR_{i/max} (%): observed inhibition efficacy; ND: Not Determined. -: no response at concentration ≤ 40 µM. *Maximum efficacy observed within the tested concentrations. ++: additive activity.