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Ecotoxicological equilibria of triclosan in Microtox, XenoScreen YES/YAS, Caco2, HEPG2 and liposomal systems are affected by the occurrence of other pharmaceutical and personal care emerging contaminants

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Abstract:

Contaminants of emerging concern may be considered as any chemicals or factors whose unintended continuous release and persistence in the environment may lead to any observable undesirable response of living beings. Still not much is known on reciprocal toxicological impact of given chemicals when present in binary or more complex mixtures. In this work, an attempt was thus undertaken to study the impact of butylparaben, methylparaben and diclofenac on toxicological behavior and properties of triclosan (at varying concentration levels) with respect to Microtox, XenoScreen YES/YAS, Caco-2, HEPG2, and liposomal systems.

Having performed analytical and biological studies modelling was done using two modelling approaches, viz., concentration addition (CA) and independent action (IA) at three concentration levels of each chemical studied. The effect of the highest concentration of triclosan studied was impacted by even small amounts of methylparaben and butylparaben in Microtox while diclofenac preferably affected triclosan activity at its lowest concentration level (with CA model). Estrogenic agonistic properties of triclosan were severely impacted by both parabens in an antagonistic way; diclofenac showed in all cases underestimation or synergy at the lowest triclosan concentration studied. Estrogenic antagonistic activity of triclosan was also slightly affected by parabens and by diclofenac in binary mixtures, showing overestimation and antagonist effects. HepG2 cells appeared to be the most resistant to the toxic effect of the mixtures at the concentrations tested and no significant proof of synergy or antagonism could be detected with the MTT assay. The liposome assays on the mixtures followed the same trends obtained with the MTT assay with Caco-2 cells, confirming the validity of the *in vitro* model used in this research. As studies on emerging contaminants mixtures toxicity are still scarce, research presented here constitute an important part in confirming utility and versatility of emerging contaminants modelling in environmental toxicology.

Keywords: triclosan toxicity, parabens, diclofenac, Microtox, endocrine potential, genotoxicity, liposomes

1. Introduction

Contaminants of emerging concern, or emerging contaminants, comprise both inorganic and organic species that are known to be persistent in the environment and are continuously introduced as a result of anthropogenic activities, either as replacements of banned chemical counterparts or owing to their positive attributes in human consumables (Schnoor 2014, Richardson and Kimura 2017). Moreover, some new chemicals of increased polarity can slip through wastewater treatment plants because of incomplete removal and ultimately enter marine settings (Reemtsma et al. 2016). The European Commission is aware of increasing amounts of unregulated chemical species in the

environment, which have potential deleterious effects on wildlife, and thus recently published the “Updated Watch List of Substances for Union-wide Monitoring in the Field of Water Policy Pursuant to Directive 2008/105/EC” (EU 2018/840). The number of chemical classes categorized as environmentally relevant emerging contaminants is steadily increasing, as highlighted by a series of comprehensive reviews (Richardson and Ternes 2014, Richardson and Kimura 2016, Richardson and Ternes 2018) and authoritative reviews (Bletsou et al. 2015, Ebele et al. 2017; La Farre et al. 2008, Lorenzo et al. 2018). These scientists are major contributors to improving the monitoring and analysis of the influence of pharmaceutical and personal care products present in natural waters, including (i) antimicrobial agents (e.g., triclosan), (ii) preservatives (e.g., alkyl esters of phthalic acid, better known as parabens), (iii) ultraviolet filters, (iv) stimulants, (v) insect repellants, (vi) artificial sweeteners, and (vii) several antibiotics, along with anti-inflammatory, anti-depressant, anti-diabetic, and anti-helminthic drugs.

Indeed, such pharmaceuticals and personal care products are among the most frequently detected pollutants in environmental settings, and their pervasiveness and continuous introduction into the trophic chain remain a major concern given their biologically active nature (Richmond et al. 2017). For example, diclofenac is a popular painkiller from the non-steroidal anti-inflammatory group, and its frequent use, especially in veterinary medicine, has led to the extinction of vultures grazing in Asian countries. The diclofenac concentration in environmental matrices varies from the nanogram to microgram per liter range (Lonappan et al. 2016). Triclosan is another common environmental contaminant, which has antifungal and antibacterial properties and is thus widely used in cosmetics, antibacterial soaps, patches, and hygienic products in hospitals. Recent studies have shown that triclosan contributes to the growth of cancer cells and can penetrate breast milk (Bever et al. 2018, Lee et al. 2014). According to studies carried out on women of different age groups, triclosan was found to accumulate in the adipose tissue, and the highest concentration was detected in women in their 30s and 40s, which is attributed to the more frequent use of cosmetics

and cleaning products in these groups. Triclosan in the environment is often detected at similar levels as diclofenac, ranging from nanograms to micrograms per liter (Allmyr et al. 2008; Petrie et al. 2016; Perez et al. 2013). In addition, methylparaben and butylparaben have been detected at the same levels in environmental samples, which are used as preservatives in cosmetics, drugs, and foods. Some studies showed that parabens have endocrine-disrupting properties and are weak estrogen receptor agonists (butyl being a stronger agonist due to its higher lipophilicity) (Okubo et al. 2001). Further details of these compounds are provided in Supplementary Table 1, and their concentrations alone and in mixtures (i.e., plausible combined toxicity) in biotic and abiotic samples are given in Supplementary Table 2.

To gain a better understanding of the actual risk posed by these chemicals in the environment, in the present study, we assessed the toxicity of binary mixtures of triclosan, methylparaben, butylparaben, and diclofenac at varying concentrations (confirmed with data presented in Supplementary Table 1 but also to set the rationale behind studies on higher order mixture and chronic toxicity studies) against organisms belonging to different trophic levels and artificial membranes. The selected organisms and organelle-like systems used as models were the bacterium *Vibrio fischeri* (Microtox[®]), genetically modified *Saccharomyces cerevisiae* yeasts (XenoScreen YES/YAS), Caucasian colon adenocarcinoma (Caco-2) and HepG2 human cell lines, and liposomes. Such approach is justified if one gets a closer look at data collected in Supplementary Table 2, which confirms impact of these chemicals to organisms belonging to all trophic levels and their organelles.

This battery of assays includes basic low-level organisms, which can be primary targets of pollutants in the environment. The gram-negative bacterium *V. fischeri* can be found in salt and brackish waters, and the change in the bioluminescence of the bacterial suspension after a period of incubation with contaminated samples forms the basis of the so-called Microtox[®] test. The XenoScreen YES/YAS test involves genetically modified yeasts harboring human androgenic (hER α)

and estrogenic (hAR α) receptors, making them sensitive to the presence of substances with endocrine properties (Marugán et al., 2012, Wieczerzak et al. 2016a). This test allows for assessments of the agonistic and antagonistic properties of chemicals in various types of samples. The human colon Caco-2 cell line is widely used in studies on the intracellular invasion of numerous chemicals and biological species; the transport of lipids, drugs, and nanoparticles; and in viral infection evaluations. HepG2 cells are an immortal cell line used to model polarized hepatocytes and their response to genotoxic, cytotoxic (including cytoprotective, anti-genotoxic, and cogenotoxic) chemicals and their toxicity (Mersch-Sundermann et al. 2004). A minimalistic *in vitro* eukaryotic cell model based on the use of large unilamellar phosphatidylcholine liposomes was also included in the present study for comparison against ecotoxicological and *in vitro* cellular assays.

Predicting the overall toxicity of mixtures containing organic chemicals, especially those with biological activity, is a very complicated task, and currently available models are associated with respective drawbacks and do not always reflect reality. Moreover, experimental studies that could provide an unambiguous answer are not always possible since it is impracticable to study all possible mixtures on various species at different concentrations to obtain actual data (Kudłak et al. 2019; Wieczerzak et al. 2016b). Among the numerous methods to assess the total impact of chemical mixtures, two main models should be differentiated: (i) the concentration addition (CA) model and (ii) the independent action (IA) model. The CA model assumes that all of the mixture components have similar modes of action (MOAs). Alternatively, in the IA model, individual components have different MOAs. These models are most often used to predict the toxicity of chemical mixtures in the field of environmental toxicology (Abendroth et al. 2011; Backhaus and Faust 2012; Belden et al. 2007; EU 2012; Gao et al. 2018; Hadrup et al. 2013; Liu et al. 2015).

As shown in Supplementary Table 2, our individual target analytes appear to have multiple effects on biota. However, their combined mode of ecotoxicity toward organisms of different trophic levels assessed with the above-mentioned modeling approaches has not been attempted to date.

Therefore, the aims of this work were as follows: (i) to determine the basic toxicity levels of analytes of interest on different biological systems and the membranotropic effects on liposomes, and (ii) evaluate the toxicological impact of the analytes when present in mixtures with two mathematical models - IA and CA. Utilizing the same standardized methodology for all assays enables obtaining reliable insights for assessing the actual risk that these mixtures pose to organisms.

2. Materials and methods

2.1. Test materials

The model compounds, namely, diclofenac sodium salt (CAS no. 15307-79-6), triclosan (CAS no. 3380-34-5, HPLC purity $\geq 97\%$), methyl 4-hydroxybenzoate (methylparaben, CAS no. 99-76-3, purity $\geq 99\%$), and butyl 4-hydroxybenzoate (butylparaben, CAS no. 94-26-8, purity $\geq 99\%$), were purchased from Sigma-Aldrich/Merck KGaA (Darmstadt, Germany). For liposome assays, 200 mM stock solutions of individual contaminants were prepared in MeOH. Binary mixtures were prepared 1000-fold concentrated in MeOH with respect to working solutions. Laurdan stock solution was 1 mM in DMSO (1000 times concentrated compared to working solutions).

For Caco2 and HepG cell assays 100 mM stock solutions of individual contaminants were prepared in a mixture of DMSO/water in order to avoid large volumes of DMSO (lower than 2% of final volume) in inhibitory curves due to the intrinsic cell toxicity of this solvent. Binary mixtures at different concentration levels were prepared in DMEM buffer from stock solutions. Although the weighted amount of every substance differed, all solutions were prepared so as to not exceed the respective solubility limits.

2.2. Microtox[®]

The Microtox[®] reagents and methodology is given in Wieczerzak et al. 2016a and in details in electronic supplementary material.

2.3. XenoScreen YES/YAS

The XenoScreen YES/YAS reagents and methodology is given in Kudłak et al. 2019 and in details in electronic supplementary material.

2.4. Cytotoxicity tests

For cell culture experiments, dimethyl sulfoxide ($\geq 99.9\%$), Dulbecco's phosphate buffered saline (PBS) at pH 7.4, Triton X-100, thiazolyl blue tetrazolium (MTT) and trypan blue powder were purchased from Sigma-Aldrich/Merck KGaA. Dulbecco's modified eagle media (DMEM; ref: 31966-021), heat inactivated fetal bovine serum (FBS), penicillin-streptomycin (PenStrep) and trypsin-EDTA were purchased from Thermo Fisher Scientific (Waltham, MA, US). Lactate dehydrogenase (LDH) Cytotoxicity Detection Kit was acquired from Takara Bio Inc. (Shiga, Japan). Caco-2 and HepG2 cell lines were purchased from the American Type Culture Collection (ATCC, LGC Standards GmbH, Wesel, Germany) and used after a passage number of 35-42, and 29-40, respectively. Absorbance readings were performed using a Synergy™ HT Multi-mode microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

The thiazolyl blue tetrazolium reduction (MTT) assay was used to evaluate cellular metabolic activity, and the lactate dehydrogenase (LDH) assay was conducted to evaluate cell viability based on changes in membrane integrity. Caco-2 and HepG2 cells (American Type Culture Collection, LGC Standards GmbH, Wesel, Germany) were used after passages 35–42 and 29–40, respectively, and cultured in Dulbecco's modified Eagle medium (DMEM; ref: 31966-021) supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 1% (v/v) penicillin-streptomycin (all from ThermoFisher Scientific, Waltham, MA, USA). The cells were maintained in a humidified chamber at 37 °C and 5% (v/v) CO₂ and were detached using 0.25% (w/v) trypsin/ethylenediaminetetraacetic acid (EDTA; ThermoFisher Scientific) when reaching 80% confluence.

Prior to the assays, each cell line was cultured in 96-well plates by adding 4×10^5 cells/mL (4×10^4 cells/well) and left to grow overnight. The supernatant was removed and replaced by 100 μ L of

the test solution (containing individual species or binary mixtures thereof) and incubated for 24 h. Thereafter, the supernatant (100 μ L) was removed for further LDH assays, and 100 μ L of MTT (Sigma-Aldrich/Merck KGaA) dissolved in a fresh culture medium (0.5 mg/mL) was added to the plate. The microplate was incubated at 37 °C for 3 h and the resulting formazan crystals were solubilized using 100 μ L of dimethyl sulfoxide (DMSO; \geq 99.9%, Sigma-Aldrich/Merck KGaA) after removal of the MTT aqueous medium. Absorbance measurements were then taken at 570 nm against 630 nm on a Synergy™ HT multi-mode microplate reader (BioTek Instruments Inc., Winooski, VT, USA). The absence of cytotoxicity (100% metabolic activity) was evaluated by replacing the test sample with the culture medium, while using 1% (v/v) Triton X-100 solution (Sigma-Aldrich/Merck KGaA) in DMEM as positive control for a cytotoxic effect. Statistical calculations were performed on the basis of the percentage of metabolic activity.

For the LDH assay (LDH Cytotoxicity Detection Kit, Takara Bio Inc., Shiga, Japan), 100 μ L of the test solutions collected over the time course of the MTT assay were centrifuged, and the supernatant was mixed with 100 μ L of the reagent mixture from the kit, comprising the catalyst (diaphorase), NAD^+ , and tetrazolium salt. After incubation for 30 min at room temperature in the dark, absorbance values were read at 490 nm and 690 nm. The absence of cell viability (100% cytotoxicity) was evaluated by replacing the test sample by 1% (v/v) Triton X-100 solution in DMEM. Statistical calculations were performed on the basis of the percentage of cytotoxicity.

The negative control for the MTT/LDH assays was performed in DMEM buffer, without any compound, showing viability values of $100 \pm 5\%$. For the positive control, 1% Triton X-100 surfactant in DMEM buffer was proven to induce cell death with viabilities of $1.00 \pm 0.05\%$. The viability values are the average of several tests and do not normally exceed 5% deviation. The toxicity of DMSO employed in the preparation of stock solutions was also evaluated. Cell viability was above 90% for the maximum concentration of DMSO in standards of individual compounds. The concentration of DMSO in binary mixtures was in all instances lower than that of the inhibition curves and thus, the solvent effect onto the toxicity is negligible.

2.5. Liposomes assay

Liposomes were prepared by lipid film hydration (Bangham et al. 1967) followed by extrusion (Hope et al. 1985). To this end, 10 mg of soybean phosphatidylcholine (PC; LIPOID S100, average molecular weight 787 g/mol) was solubilized in 1.0 mL of chloroform. The solvent was first removed in a rotary evaporator at low pressure (290 mbar) and 30 °C for 2 h and then under vacuum for approximately 2 h or more so as to obtain a uniform dried lipid film without any organic solvent traces. The lipid was then reconstituted in 1 mL phosphate buffered saline (PBS, pH 7.4; Sigma) at room temperature to afford a final lipid concentration of approximately 12.7 mM PC, which was then vortexed for 1 h (1 min every 5 min) to obtain multilamellar vesicles (MLVs). The milky suspension was maintained overnight at 4 °C to allow the MLVs to become sufficiently hydrated for stabilization. Subsequently, the solution was extruded 29 times through polycarbonate filters (100-nm pores) to generate highly homogeneous large unilamellar vesicles with a hydrodynamic diameter (Z-average) of 129 nm and a polydispersity index as low as 0.076. Liposomal characterization was performed by dynamic light scattering using a Zetasizer Nano ZS90 system (Malvern Panalytical, Malvern, UK).

The effect of the individual contaminants and mixtures on the lipid packing and ordering of liposomes was investigated by a membrane fluorescence probe, *v6*-dodecanoyl-*N,N*-dimethyl-2-naphthylamine (Laurdan). The Laurdan fluorescence emission spectrum is strongly dependent on the polarity of its surrounding medium once inserted into the lipid membrane; the higher the concentration of water (or another polar solvent) in the vicinity of the probe, the higher the red shift of the spectrum from 440 nm towards 490 nm will be. This effect is caused by the so-called dipolar relaxation phenomenon and is a consequence of the decrease of the probe's excited state energy due to reorientation of the molecules of the polar solvent around the probe's dipole (Parasassi et al. 1986). This phenomenon is measured by the generalized polarization (GP) parameter that correlates well with the phase state of the membrane (Parasassi et al. 1991). In the gel phase, the membrane is more ordered and thus less hydrated, and the GP takes on positive numbers; otherwise, for a

disordered and hydrated membrane, the so-called liquid-crystalline phase (as is the case in this work), the GP values are negative. GP values were obtained using the following equation:

$$GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}} \text{ (eq. 1.)}$$

where I_{440} and I_{490} represent the emission intensities of the liposome-embedded Laurdan at 440 nm and 490 nm, respectively. GP values range from +1 to -1.

For experimental GP measurements, liposomes, obtained as described above, were diluted in PBS (pH 7.4) at a final concentration of 100 μ M PC. The membrane probe (stock solution of 1 mM in DMSO) was added to the liposomal solution at a final concentration of 1 μ M Laurdan and kept in the dark at room temperature for 2 h to allow for equilibration of the probe into the membrane (Oliver et al. 2016). The liposomal solution was then divided into 1-mL aliquots. One microliter of the emerging contaminants, alone or as binary mixtures in methanol, was added to the 1-mL aliquots of the Laurdan-embedded liposomes. Samples were incubated for 30 min at room temperature and placed in a 96-well white microplate with a solid bottom (cat. no. 30196, SPL Life Sciences) for further fluorescence detection. Fluorescence data were recorded by a Varian Cary Eclipse fluorescence spectrometer (Agilent technologies, Mulgrave, Victoria) at room temperature. The emission intensity was measured at 440 nm and 490 nm (excitation wavelength was fixed to 360 nm). Operational parameters were set to 10-nm slit widths and a 600-V photomultiplier detector voltage.

The $GP_{\text{contaminant}}/GP_{\text{Laurdan}}$ ratio was used as the analytical parameter for statistical calculations. Using the Laurdan GP as a control, $GP_{\text{contaminant}}/GP_{\text{Laurdan}}$ percentage ratios close to 100% indicated the lack of effects of contaminants on the morphology and structural characteristics of the liposomes (i.e., absence of membrane toxicity). However, lower or higher GP percentages indicated a greater membranotropic effect, serving as a marker of the potential toxicity of the contaminant and/or binary mixture. The negative control for liposome assays was carried out only with the

fluorescent probe Laurdan to get the initial fluorescence signal or GP value that serves as a reference. We assigned the value of 100% “viability” or 100% of no effect to this signal. There is no positive control in this case because the value of 0% does not make sense as all the experimental values are relative to the GP of Laurdan alone. Methanol in the working solution not exceeded 0.1% so solvent control was not considered as relevant in terms of liposome stability.

2.6. Apoptosis and CYP450-based assays for HepG2 cells

The apoptotic effect of the different chemicals on HepG2 cells was evaluated using FITC Annexin V Apoptosis Detection Kit I (BioLegend, San Diego, CA, USA) according to the manufacturer instructions. In brief, after incubation with the contaminant mixtures for 24 h, the cells were washed twice with PBS, detached with 0.25% trypsin-EDTA, and centrifuged for 5 min. Subsequently, the supernatant was discarded, the cells were resuspended in 50 mL of binding buffer with Annexin V-FITC and 7-AAD reagents, and incubated 15 min prior to analysis using a BD Accuri C6 flow cytometer (BD Biosciences, Erembodegem, Belgium). For each sample, a minimum of 20 events were recorded.

The effect of the different chemicals on expression of the CYP450 family was also assessed by flow cytometry. HepG2 cells were labeled with cytochrome P450 1A2-Alexa Fluor 647 and cytochrome P450 3A4-FITC antibodies (Novus Biologicals, Centennial, CO, USA) according to manufacturer instructions, and then analyzed with the BD Accuri C6 flow cytometer.

2.7. Model deviation ratio (MDR) analysis

The IA and CA models were used to calculate the actual toxic effect of the tested binary mixtures. These two approaches serve to assess the combined toxicological effect of chemicals assuming a similar MOA (CA) or dissimilar MOA (IA). CA models are more frequently applied for environmental risk assessments because they are slightly more conservative than IA models. The combined toxicological effect of a mixture assessed by the CA model is given by equation 2:

$$ECx_{mix} = \left(\sum_{i=1}^n \frac{p_i}{ECx_i} \right)^{-1} \quad (\text{eq. 2.})$$

where EC_{mix} is the x_{mix} effect caused by the total concentration of the mixture of studied chemicals (components) (Expected value), p_i indicates the proportion of component i in the mixture calculated based on the concentration of component i in the mixture, n indicates the number of components in the mixture, and ECx_i indicates the x_i effect caused by component i at a given studied concentration in the mixture.

The CA model does not account for possible interactions between different chemicals in the mixture, and deviations of the tested mixture toxicity from the predicted values could be evidence of synergistic or antagonistic interactions between chemicals.

By contrast, IA models assume independent action from the combined chemicals, and were calculated using equation 3:

$$E(C_{mix}) = 1 - \prod_{i=1}^n (1 - E(c_i)) \quad (\text{eq. 3.})$$

where EC_{mix} is the overall effect expressed as a fraction of the maximal possible effect of a mixture of chemical i in a mixture with n chemicals (expected value), c_i indicates the concentration of component i in the mixture, n indicates the number of components in the mixture, and $E(c_i)$ indicates the effect of component i , applied separately.

To confirm/deny the presence of synergy/antagonism, the MDR coefficient was used to compare predicted and observed values (Kudłak et al. 2016, Wiczerzak et al. 2015). MDR is defined as shown in equation 4:

$$\text{MDR} = \frac{\text{Expected}}{\text{Observed}} \quad (\text{eq. 4.})$$

where *Expected* is the effective toxicity of the mixture predicted by the CA or IA models, and *Observed* is the effective toxicity of the mixture obtained during the toxicity assessments.

Mixtures with $\text{MDR} > 2.0$ are considered to exhibit a high probability of antagonism, while those with values below 0.5 show a synergistic characteristic. Here, we arbitrarily assumed that an

MDR falling within 0.50–0.71 and 1.40–2.00 indicates possible under- and overestimation of the models, respectively.

3. Results and discussion

Inhibition curves were performed in a concentration range based on previous experiments and the know-how of our group. After obtaining IC_{50} values of every single compound, triclosan exhibited the major toxicity, with the lower IC_{50} , namely, ten times lower than butylparaben, the second most toxic compound of our selected contaminants. In this way, it was decided to evaluate the effect of the other contaminants on the toxicity of triclosan throughout binary mixtures using the IC_{20} value of triclosan at 100% IC_{20} , 66% IC_{20} and 33% IC_{20} . IC_{20} was chosen instead of IC_{50} for different reasons: i) to diminish working concentrations of pollutants, thus being closer to environmental values; ii) to avoid high levels of dead cells, as the effect of binary mixtures on cell viability is unknown due to possible synergistic effects that could increase the toxicity to close 100%, and thus discrimination of the effects of different binary mixtures would be impracticable.

3.1. Microtox[®]

The results of CA modeling with bioluminescent bacteria demonstrated intriguing trends. The highest concentration of triclosan was impacted by methylparaben to the greatest extent with a decreasing content of methylparaben; the same behavior was evident in the case of the mixture of triclosan with butylparaben, showing synergistic toxicity effects. The trend of the diclofenac impact was similar but with a much lower magnitude. In the case of mixtures containing the lowest concentrations of triclosan, the CA model with methylparaben indicated no synergism or antagonism and suggested underestimation for butylparaben and diclofenac. IA models showed that in most cases, the analytes have independent MOAs because there is a good agreement between theoretical and experimental data.

In fact, independent MOAs have also been observed indirectly for *Mytilus galloprovincialis* mussels exposed to triclosan and diclofenac in different salinity conditions (Freita et al. 2019). The

metabolic capacity and oxidative status were changed in all tested conditions, but triclosan had effect over glutathione peroxidase activity while exposure to diclofenac enhanced catalase response.

Table 1 shows the variations in MDR values depending on triclosan concentrations for solutions of selected parabens and diclofenac evaluated with Microtox. Overestimation of the IA model was only detected for mixtures of triclosan and methylparaben at the lowest concentrations tested for these compounds.

Table 1.

3.2. XenoScreen

3.2.1. XenoScreen YES+

The estrogenic agonistic behavior of analytes can confirm behavior related to the endocrine disruption potential of mixtures. As shown in Table 2, both the CA and IA models confirmed an antagonistic impact of methyl- and butylparaben on triclosan. Moreover, a concentration-dependent impact was noticeable in which reducing the concentration (down to the 1-micromolar level) of substances in the mixtures enhanced the antagonistic impact concerning their interactions. In this context, considering a recent study concerning prenatal exposure to certain xenobiotics, including triclosan and methyl-, butyl- and propyl-paraben, adverse birth outcomes have been associated with these compounds. Triclosan was positively associated with gestational age among males, and negatively associated with gestational age among females. Methyl-, butyl- and propyl-paraben were associated with decreased odds of small-for-gestational age infants, thus showing, in a population-based study context, that these compounds may have different endocrine effect (Aker et al. 2019). This topic has also been summarized in a review concerning the impact of environmental exposure to endocrine disrupting chemicals on the reproductive potential among women (Karwacka et al. 2019), measured by ovarian reserve and by assisted reproductive technology outcomes. Exposure to triclosan decreased the oocyte quality and the embryo quality while exposure to parabens decreased the antral follicle count and the rate of clinical pregnancy and live births. A

recent study also evaluated the associations between repeated measures of several chemicals and their mixtures with reproductive hormones in women (Pollack et al. 2018). Parabens were associated with increased estradiol while phenols (including triclosan) were associated with decreased estradiol, luteinizing hormone, and follicle stimulating hormone, showing also here an antagonistic effect.

The observed impact of diclofenac on the endocrine potential of triclosan was even more interesting from an environmental point of view in which increasing concentrations of diclofenac (while keeping the concentration of triclosan constant) suggested a possible synergistic impact of the mixtures (MDR values reached down to 0.71).

Table 2.

3.2.2. *XenoScreen YES-*

The estrogenic antagonistic effect (*XenoScreen YES-*) of triclosan was only slightly impacted by the co-presence of the other analytes tested (Supplementary Table 3). Interestingly, some behavioral trends were noted in which butylparaben moderately impacted the endocrine potential of triclosan at low and medium concentrations based on CA models. IA calculations however showed a tendency of diclofenac to exhibit a certain antagonistic impact, especially at higher concentrations of the drug. Taking into account the most plausible scenario of the independent MOAs of these analytes, it can be concluded that diclofenac does mitigate the *YES-* based triclosan toxicity.

3.2.3. *XenoScreen YAS+*

The androgenic agonistic behavior of triclosan mixtures with methyl- and butylparaben were very well modeled with the CA approach, whereas IA modeling values showed antagonistic action for many of these mixtures (MDR values > 2.0) or overestimation (Supplementary Table 4). Interestingly, in the mixture of triclosan with diclofenac, synergy was observed more often with CA modeling (and underestimation), which was confirmed by IA mathematical calculation. At the lowest concentration

of triclosan tested, potentiation of synergy with diclofenac was observed, confirming the great threat of these chemicals when present in the environment in combination at concentrations that would otherwise be considered non-toxic on their own.

3.2.4. XenoScreen YAS-

The antagonistic androgenic behavior of triclosan was barely affected by methyl- and butylparabens. No such activity was confirmed in the MDR analysis (Supplementary Table 5), although IA modeling indicated some overestimation results. By contrast, for diclofenac, both overestimation and antagonism were observed in almost all mixtures with triclosan based on the CA model and in all cases calculated with the IA model. This result is of particular interest considering the plausible synergy of such mixtures from the YES+ analysis, demonstrating that triclosan-diclofenac mixtures at environmentally stated levels showed both anti-androgenic and pro-estrogenic activities.

3.3. MTT and LDH assays of Caco-2 and HepG2 cells

Prior to the cytotoxicity testing of xenobiotic mixtures, inhibition curves of each compound were constructed after 24 h of exposure. In accordance with our previous findings summarized above, triclosan again emerged as the most toxic pollutant for both cell lines, with 20% inhibitory concentration (IC_{20}) values of 150 μ M and 72 μ M for Caco-2 and HepG2 cells, respectively. Based on the IC_{20} value for the discrimination of potential toxic effects and the reliable measurement of potential synergy across mixtures, three triclosan concentrations were defined as C3, C2, and C1, corresponding to 100%, 66%, and 33% of the IC_{20} at 150, 100, and 50 μ M for Caco-2 cells, and 72, 48, and 24 μ M for HepG2 cells. The inhibition curves revealed non-toxic effects for the other contaminants at the concentrations chosen for triclosan. Therefore, the effects of methylparaben, butylparaben, and diclofenac on triclosan were evaluated in all cases at the same C3, C2, and C1

levels (see the SI) with all possible combinations to determine how these xenobiotics may interact and alter the toxic action of triclosan. HepG2 cells appeared to be the most resistant to the toxic effect of the mixtures at the tested concentrations of xenobiotics (Supplementary Table 6). None of the concentration mixtures showed significant indices of synergy or antagonism based on the MTT assay. Only the binary mixtures of triclosan with butylparaben or diclofenac, all at the highest level (C3), showed a trend towards synergic effects on these cells. This finding is in good agreement with previous results of Rudzok et al. (2010) who reported synergism for the combination of triclosan and diclofenac by MTT assays using both the IA and CA models.

Results of the MTT assay for Caco-2 cells confirmed lack of a clear impact of the parabens studied on the toxicity of triclosan in a binary mixture. In both the CA and IA models, there were no indications of synergy or antagonism (Supplementary Table 7). There was only one case of strong synergy observed when the highest concentrations of triclosan and butylparaben were tested. However, further validation of this result in subsequent studies with a larger number of intervals within the studied range is warranted, as the trends of both the IA and CA models suggest that this behavior is plausible but the abrupt change from lack-of-impact to synergy appears too strong under the tested intervals. Moreover, there were interesting results found for the mixture of triclosan with diclofenac with both the CA and IA models, demonstrating evident cases of underestimation and synergy for the highest concentration of triclosan in combination with C2 and C3 levels of diclofenac. This further justifies the need of additional studies of the impact of triclosan on these pharmaceuticals at environmentally relevant levels in risk assessment explorations. In fact, a recent study targeting the evaluation of the delayed toxicity during the developmental stage on zebrafish embryos showed that a mixture of 8 pharmaceuticals at their highest environmental concentration levels had an adverse effect on larval behaviour during embryonic development (Zhou et al. 2019). Experiments using each compound alone did not elicit this negative response, stressing the importance of evaluating combinations of compounds.

LDH assays were performed only for the most cytotoxic mixtures according to the previous MTT results; that is, C3 of triclosan mixed with C1, C2, and C3 of both butylparaben and diclofenac. While the MTT test is indicative of cell metabolic activity, the LDH assay can be used as a cell death marker because this cytoplasmic enzyme is released into the surrounding environment when the cell membrane is damaged. For Caco-2 cells, binary mixtures of triclosan and butylparaben showed a strong synergistic effect on cytotoxicity (cell death) as the concentration of the latter pollutant increased, which was in line with the results of the MTT assay (see above). Further, there was no appreciable change of cytotoxicity for mixtures of triclosan with diclofenac at any concentration level of both contaminants. These differences further suggest that the mechanisms of the interaction of butylparaben or diclofenac with triclosan on live cells are somewhat different. Environmental concentrations of diclofenac, methylparaben and butylparaben did not show any effect on the cell toxicity of triclosan at the IC₂₀ value (100%, 66% and 33%). Environmental concentrations were in the range of ng/L (pM) against the IC₂₀ triclosan at the mg/L (μM) level. The range of environmental concentrations selected for the studies is based on the following references: 5.7-62 ng/L MP (Jonkers et al. 2010), 0.2-7.1 ng/L BP (Jonkers et al. 2010), 60-460 ng/L DCF (McEneff et al. 2014).

The combination of triclosan with butylparaben or diclofenac did not show any trend in cell death for the hepatocytes, thus indicating that the behavior of the contaminant mixtures on HepG2 cells is entirely different than that on Caco-2 cells. The experimental results demonstrated a low degree of cell damage for the mixtures of PPCPs, which is in good agreement with the detoxifying ability of hepatocytes towards xenobiotics.

3.4. Liposome tests

The three concentration levels of each of the four investigated xenobiotics were set at 24, 48, and 72 μM, according to the data used above for the cytotoxicity tests, wherein the IC₂₀ value of

triclosan for the hepatocyte cell line was 72 μ M. Notably, the liposome tests on the mixtures followed the same trends obtained with the MTT assay with Caco-2 cells (Supplementary Table 8), thus indicating the validity of the *in vitro* model. Methyl- and butylparaben showed a negligible impact on the toxicity of triclosan at any of the concentration levels studied (although butylparaben seemed to have a slightly higher relative impact on the toxicity of triclosan than methylparaben). As shown in Supplementary Table 8, the toxicity of triclosan was strongly affected by diclofenac, mathematically confirming that synergy of such a binary mixture occurs at the highest concentration levels studied.

3.5. CYP expression and apoptosis

Metabolism determines the fate of a compound upon entry in the human body. Among the various drug metabolizing enzymes, cytochrome P450s (CYP450) constitute an important protein family acting in xenobiotic metabolism, which usually converts xenobiotics into safe metabolites. This activity can be detected by an increase in the gene and protein expression. As CYP 450 xenobiotic-metabolizing enzymes are primarily expressed in the liver, HepG2 cells were studied (Burkina et al. 2017). No enhancement in the expression of CYP450 3A4 and CYP450 1A2 was observed for HepG2 cells under the selected experimental conditions (highest concentrations of triclosan, diclofenac, and butylparaben; mixtures of triclosan and diclofenac; and mixtures of triclosan and butylparaben at the levels specified above for HepG2 cells), indicating that the occurrence of these pollutants and their mixtures did not cause the induction of CYP expression. This finding is in good agreement with previous studies demonstrating no effect of triclosan on CYP-mediated metabolism in either HepG2 cells or HepG2/vector cells (Wu et al. 2017).

Apoptosis was observed in some conditions, namely in the presence of the highest concentration of triclosan (46.4% apoptotic cells with triclosan at C3). The presence of butylparaben

in combination with triclosan seemed to inhibit apoptosis in an inverse-concentration-dependent manner. Specifically, similar values for apoptotic cells were found for the highest concentration of butylparaben (46.6%), and the apoptosis frequency decreased when lower concentrations of butylparaben (C1 and C2) were concomitantly present with the highest concentration of triclosan (C3) in the reaction medium (40.1% for C2 butylparaben and 28.4% for C1 butylparaben).

4. Conclusions

Environmental toxicity testing has traditionally mainly focused on the effects of single pollutants only (Wieczerek et al. 2016b); however, the combinations of common contaminants in a given environment can have very different effects than their independent impacts. The present study contributes to and expands recent efforts in this field to find the best modeling approaches for describing the impact of compounds (when present in mixtures) in selected bioassays. The need for such comprehensive analyses is becoming increasingly apparent in modern ecotoxicological studies. In the present work, we confirmed a significant impact of selected paraben compounds with varied log P values and diclofenac on the ecotoxicological potential of triclosan, a common antimicrobial agent used in numerous applications. As shown by the MDR analysis of CA and IA models, the type of interaction (or its lack) is mainly dependent on the concentration levels of the various contaminants tested. This justifies the importance of considering results from mixtures in ecotoxicological studies when describing and assessing the environmental impact of chemicals, whereas the majority of previous cytotoxicity tests of triclosan have not considered its impacts in mixtures (Zhang et al. 2019a, Ma et al. 2013, Wang et al. 2019). In fact, such ecotoxicity data of mixtures should be also considered by policy makers and authorities issuing admissible concentration levels of different substances found in everyday products or medications.

Based on our results and a review of the relevant literature, it is clear that ecotoxicity assessments require testing a wide range of concentration levels of various chemicals (e.g., BPA analogues, heavy metals, PPCPs, PAHs) in both their binary mixtures and higher-order combinations. Indeed, mixtures of chemicals clearly behave differently on biological systems than each of the components individually. However, more research is needed in this regard, focusing not only at the levels recorded in different environmental or biological settings but also at lower levels for which no toxicological response would be theoretically expected within a given timeframe (different for acute or chronic toxicity studies). A current trend in this field is the development of fast and accurate online screening methodologies to shorten the response times of currently used bioassays and enable the rapid confirmation of the existence/lack of synergy/antagonism between mixture constituents (and also their degradation products).

The most reliable approach to tackle this problem should involve the development of fast and accurate online screening methodologies to shorten the response times of currently used bioassays and enable the rapid confirmation of the existence/lack of synergy/antagonism between mixture constituents (and their degradation products). The present study provides clear confirmation of the impact of diclofenac, methylparaben, and butylparaben on the toxicity of triclosan to almost all organisms and systems studied, demonstrating that even low concentrations of these contaminants may trigger undesirable effects on organisms of different trophic levels.

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Table 1. MDR values depending on the triclosan concentration for solutions of selected parabens and diclofenac studied with Microtox (MDR values >2.0 exhibit antagonism, MDRs <0.5 show synergism, MDR values within 0.50-0.71 and 1.40-2.00 values mean, respectively, under- and overestimation of presented models; for values of particular concentrations C1, C2 and C3 of all analytes please refer to the table below)

CA						IA		
Triclosan ^a						Triclosan ^a		
C1	C2	C3				C1	C2	C3
0.71	0.87	1.01	C1	^b Methylparaben	C1	1.08	1.18	1.16
0.60	0.79	1.01	C2		C2	1.09	1.25	1.23
0.40	0.69	1.00	C3		C3	1.17	1.64	1.50
0.68	0.93	0.65	C1	^c Butylparaben	C1	1.05	1.36	0.91
0.59	0.66	0.47	C2		C2	1.11	1.15	0.72
0.27	0.31	0.51	C3		C3	0.74	0.78	0.93
0.85	0.83	0.71	C1	^d Diclofenac s.	C1	1.15	1.15	1.07
0.72	0.78	0.57	C2		C2	1.09	1.21	0.91
0.66	0.72	0.70	C3		C3	1.07	1.22	1.21

Percentile values for model deviation ratios (MDR) and numbers of cases for each group of CA and IA experiments of BP toxicity studies

Model	no. of cases				percentile			
	Synergism	Under-estimation	Over-estimation	Antagonism	80	90	95	99
	CA	4	11	0	0	0.846	0.958	1.007
IA	0	0	2	0	1.218	1,294	1,458	1,604

^a Triclosan			^b Methylparaben			^c Butylparaben			^d Diclofenac sodium salt			μM
C1	C2	C3	C1	C2	C3	C1	C2	C3	C1	C2	C3	
0.389	0.778	1.180	17.10	34.30	51.90	1.30	2.60	3.94	24.1	48.2	73.0	

Table 2. MDR values depending on the triclosan concentration for solutions of selected parabens and diclofenac studied with XenoScreen YES+ (MDR values >2.0 exhibit antagonism, MDRs <0.5 show synergism, MDR values within 0.50-0.71 and 1.40-2.00 values mean, respectively, under- and overestimation of presented models; for values of particular concentrations C1, C2 and C3 of all analytes, please refer to the table below)

CA						IA		
Triclosan ^a						Triclosan ^a		
C1	C2	C3				C1	C2	C3
3.87	3.20	2.52	C1	^b Methylparaben	C1	7.02	6.28	5.43
2.71	2.64	1.83	C2		C2	4.70	4.89	3.47
2.64	2.38	1.03	C3		C3	4.90	4.65	1.91
2.91	3.27	2.08	C1	^c Butylparaben	C1	8.06	9.11	6.90
3.33	2.95	1.95	C2		C2	7.26	6.71	5.14
2.61	2.93	0.87	C3		C3	5.34	6.40	2.20
0.62	0.71	0.59	C1	^d Diclofenac sodium salt	C1	1.70	1.48	1.21
0.60	0.59	0.59	C2		C2	1.70	1.27	1.25
0.42	0.58	0.58	C3		C3	1.26	1.33	1.30

Percentile values for model deviation ratios (MDR) and numbers of cases for each group of CA and IA experiments of BP toxicity studies								
Model	no. of cases				percentile			
	Synergism	Under-estimation	Over-estimation	Antagonism	80	90	95	99
	CA	1	8	2	14	2,926	3,228	3,312
IA	0	0	4	17	6,648	7,116	7,820	8,837

^a Triclosan			^b Methylparaben			^c Butylparaben			^d Diclofenac sodium salt			μM
C1	C2	C3	C1	C2	C3	C1	C2	C3	C1	C2	C3	
12.5	31.3	50.0	0.45	1.13	1.81	0.039	0.097	0.155	5.8	14.4	23.0	

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Highlights

- Mixtures of emerging pollutants impact the organisms of all trophic levels.
- Estrogenic agonistic properties of triclosan are antagonistically affected by parabens.
- HepG2 cells were the most resistant to the toxic effect of the mixtures studied.

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