Generalized concentration addition accurately predicts estrogenic potentials of mixtures and environmental samples containing partial agonists

Markus Brinkmann[a,b,*], Markus Hecker[a], John P. Giesy[c,d,e], Paul D. Jones[a], Hans Toni Ratte[b], Henner Hollert[b,f,g,h,i], Thomas G. Preuss[b,1]

[a] *School of Environment & Sustainability and Toxicology Centre, University of Saskatchewan, 44 Campus Drive, Saskatoon, SK, S7N 5B3, Canada*
[b] *Institute for Environmental Research, RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany*
[c] *Department of Veterinary Biomedical Sciences and Toxicology Centre, University of Saskatchewan, 44 Campus Drive, SK S7N 5B3 Saskatoon, Canada*
[d] *Department of Zoology and Center for Integrative Toxicology, Michigan State University, East Lansing, MI, USA*
[e] *School of Biological Sciences, University of Hong Kong, Kowloon, Hong Kong, SAR, China*
[f] *College of Resources and Environmental Science, Chongqing University, 1 Tiansheng Road Beibei, Chongqing 400715, China*
[g] *College of Environmental Science and Engineering, Tongji University, 1239 Siping Road, Shanghai, China*
[h] *State Key Laboratory of Pollution Control and Resource Reuse, Tongji University, 1239 Siping Road, Shanghai, China*
[i] *State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, China*

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ABSTRACT

Cell-based bioanalytical tools are considered one alternative to overcome limitations of sensitivities of instrumental, analytical chemistry for monitoring estrogenic chemicals in the environment. Because these tools also reflect non-additive interactions of chemicals in mixtures, their outcomes often deviate from outcomes of chemical analytical approaches that assume additivity, e.g. the concentration addition (CA) model. Often this is because CA is unable to adequately represent effects of partial agonists, i.e. estrogens with lesser efficacies compared to 17β-estradiol. A generalized concentration addition (GCA) model has been proposed to address this shortcoming. In the present study, we investigated effects of mixtures of isomers of nonylphenol as partial model agonists in a cell-based estrogenicity assay. Whether the GCA model was able to more accurately predict the outcomes of these and previously published mixture experiments was evaluated, as well as the potency of a set of comprehensively characterized sewage effluent samples, compared to CA. If samples contained partial agonists, the GCA model consistently predicted potencies of mixtures and extracts of environmental samples more accurately than did the CA model. These findings enable more accurate estimations of potencies of estrogenicity explained by concentrations of agonists and partial agonists, thus significantly improving the ability to identify causative chemicals.

1. Introduction

An ever-increasing number of chemicals are released into aquatic environments through various sources. Some of these chemicals are monitored on a routine basis for their well-understood effects on aquatic organisms, and potentially even on humans. Some compounds, however, have only recently been detected due to development of more sensitive and unbiased non-target analytical screening methods (Petrović et al., 2003). These chemicals of emerging concern (CECs) can interfere with a plethora of biological functions in aquatic organisms and ultimately even humans (Bolong et al., 2009; Snyder et al., 2003; Triebskorn et al., 2013). Some CECs, referred to as endocrine-disrupting chemicals (EDCs), can potentially disrupt neuro-endocrine functions in exposed organisms. One particularly well-studied class of EDCs are natural and synthetic estrogens, which can bind to the estrogen receptor (ER) and have been shown to interfere with the hypothalamus-pituitary-gonad($-$liver) (HPG[L]) axis of aquatic vertebrates, particularly fish. Fish are sensitive to these compounds: Effects on
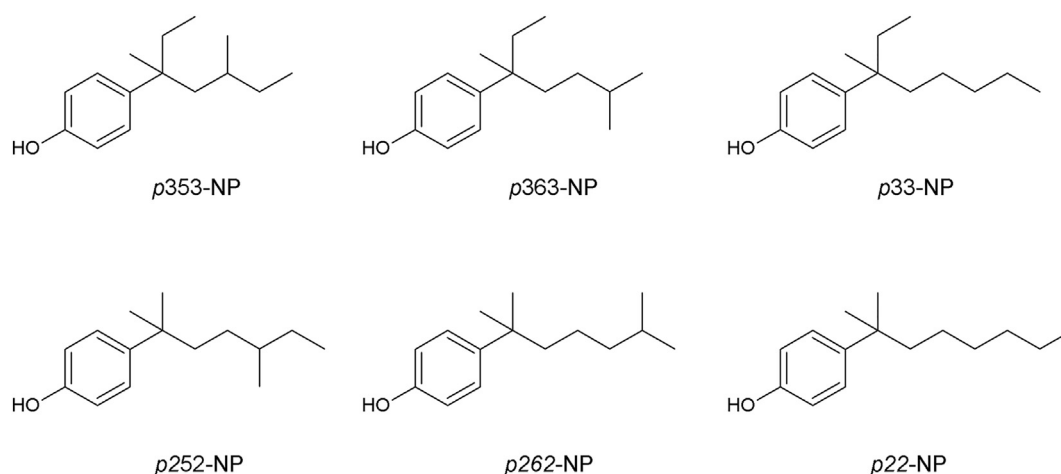
Fig. 1. *p*-NP isomers investigated in the present study (cf. Preuss et al., 2006).

reproduction occur at concentrations in the low ng L^{-1} range (Ankley et al., 2001; Hecker et al., 2002; Jobling et al., 1996; Leino et al., 2005; Pawlowski et al., 2004). Previously unexposed populations of wild fishes in the Experimental Lakes Area of Ontario, Canada, collapsed after exposure to environmentally relevant concentrations (5 ng L^{-1}) of the synthetic estrogen 17α-ethinylestradiol (EE2) (Kidd et al., 2007; Kidd et al., 2014). Although analytical equipment capable of detecting EDCs at such small concentrations is becoming more and more available, many xenoestrogens cannot be routinely monitored with the instruments commonly available to monitoring authorities (Wernersson et al., 2015).

To overcome limitations of instrumental analytical chemistry, cell-based bioanalytical tools have been developed as cost-effective and sensitive alternatives (Brack et al., 2017; Di Paolo et al., 2016; Kunz et al., 2017; Wernersson et al., 2015). These assays make use of specific and quantitative responses of wild-type or recombinant reporter gene cell lines to exposure with specific classes of chemicals (Behnisch et al., 2002; Coors et al., 2004; Eichbaum et al., 2014). One advantage of such bioanalytical tools is that they integrate effects of all chemicals in a mixture, which exert their activity through the same mechanism of action (e.g. binding to the ER), and not only those for which instrumental methods exist to allow their quantifications (Otte et al., 2013). Potencies of mixtures or extracts of environmental samples are then expressed relative to the response to a standard. For example, when characterizing exposures to estrogenic chemicals responses of bioassays would be normalized to the potent, endogenous estrogen 17β-estradiol (E2), and expressed as E2 equivalent concentrations (EEQs) per unit volume or weight (e.g., ng L^{-1} or ng kg^{-1}). Potencies of natural and synthetic estrogens are commonly expressed as E2 equivalency factors (EEFs), i.e. the ratio of half-maximal effect concentrations (EC$_{50}$s) of the E2 standard and the chemical (Körner et al., 1999) or the point of departure of the concentration-response relationship, represented by the EC$_{20}$. In mass-balance analyses of complex environmental mixtures, these EEFs are often used to calculate EEQs by means of a special form of the concentration addition (CA) model (Hadrup et al., 2013; Pojana et al., 2007; Eq. (1)). This so-called EEF approach, however, is based on a central assumption: That all estrogenic compounds in the mixture differ only in their potencies; or said another way, that their efficacies (i.e. the maximum achievable effect level) and slopes of the concentration-response curves are equal. While these assumptions work reasonably well for mixtures that are dominated by full agonists, e.g. steroidal estrogens, they result in significant over-estimation of the potency of mixtures that contain partial agonists (i.e. compounds with efficacies significantly less than that of E2), e.g. different nonylphenol (NP) isomers (Howard and Webster, 2009; Preuss et al., 2010).

$$EEQ = \sum_{i=1}^{n} c_i EEF_i \tag{1}$$

where: *EEQ* is the E2 equivalent concentration of a mixture of *n* individual chemicals with known E2 equivalency factors (*EEF$_i$*) at concentrations *c$_i$*.

As a potential solution to this problem, an extended and generalized concentration addition (GCA) model has been proposed for a different group of contaminants (Howard and Webster, 2009). This model accounts for differences in efficacy and has been effectively applied to predict mixture effects of partial and full agonists of the aryl hydrocarbon receptor (AhR) (Howard et al., 2010), and of chemicals affecting steroid synthesis in the H295R assay (Hadrup et al., 2013). An evaluation of the potential use of GCA to describe potencies of mixtures of partial and full agonists of the ER, and to predict the potency of extracts of environmental samples containing partial agonists, however, has not been reported to date. To address this limitation, this study generated a dataset for effects of two tertiary and one quaternary mixture of isomers of nonyl-phenol (NP) in a cell-based estrogenicity assay. Furthermore, we evaluated whether GCA was able to more accurately predict the outcomes of these and other previously published mixture experiments (Preuss et al., 2010), as well as the potency of a set of comprehensively characterized sewage effluent samples that was available from the literature (Körner et al., 2001; Spengler et al., 2001), compared to CA.

2. Materials and methods

2.1. Chemicals

Linear 4n–NP (99% purity) was commercially available from Avocado, Germany. The six isomers 4-(3′,5′-dimethyl-3′heptyl)phenol (*p*353-NP), 4-(3′,6′-dimethyl-3′heptyl)phenol (*p*363-NP), 4-(2′,5′-dimethyl-2′heptyl)phenol (*p*252-NP), 4-(2′,6′-dimethyl-2′heptyl)phenol (*p*262-NP), 4-(3′-methyl-3′octyl)phenol (*p*33-NP), and 4-(2′-methyl-2′octyl)phenol (*p*22-NP) were synthesized as described previously (Ruß et al., 2005; Vinken et al., 2002; Fig. 1). After synthesis, isomers were purified by means of silica gel column chromatography with hexane:ethylacetate (1:13, *v*/v) as the elution solvent. Purities of the isomers were determined using gas chromatography–mass spectrometry (GC/MS) and in all cases exceeded 99.9%. Stock solutions were prepared in ethanol and stored at −20 °C. As determined by GC/MS, isomers were stable at least for 2 years under these storage conditions.

2.2. MVLN assay

The MVLN assay uses MCF-7 human breast carcinoma cells that

have been stably transfected with a luciferase reporter gene. Expression of luciferase, which can be quantified through emission of light in the presence of the substrate luciferin, is proportional to binding of ligands to the ER. Detailed protocols for culture of MVLN cells and test procedures have been published elsewhere (Preuss et al., 2006; Snyder et al., 2001). Briefly, MVLN cells were cultured in Dulbecco's Modified Eagle Medium with Hams F-12 nutrient mixture (Sigma-Aldrich, Germany), containing 1 mM sodium pyruvate (Sigma-Aldrich), 1 mg L^{-1} insulin (Sigma-Aldrich) and 10% (v/v) dextran-charcoal stripped fetal bovine serum (FBS, Hyclone, Logan, UT). Cells were incubated at 37 °C in an atmosphere enriched with 5% CO$_2$ (v/v). For exposure experiments, 250 μL of a suspension of MVLN cells (7.5 10^4 cells mL^{-1}) were seeded into individual wells of a standard 96-well microplate.

Concentration-response curves for the six NP isomers and results of co-exposures of graded concentrations of NP isomers with 100 and 1000 pM E2, respectively, have been reported previously (Preuss et al., 2006; Preuss et al., 2010). Additional experiments were conducted in which graded concentrations of mixture (p353/p363/p262-NPs, p353/p33/p252-NPs and p353/p363/p33/p262-NPs, respectively) were tested at fixed equal concentration ratios of each chemical. An E2 standard (4.1 to 1000 pM) and a protein standard curve (bovine serum albumin, Sigma-Aldrich) were included during each test. The concentration of ethanol did not exceed 1% (v/v) in any case. Luciferase activity was measured following 72 h incubation under standard culture conditions in a plate luminescence reader (Spectrafluor Plus, Tecan, Männedorf, Switzerland) after addition of LucLite cocktail (Packard Instruments, Meriden, USA). To account for cytotoxicity, a fluorescamine-based assay was used to measure the protein content in each well.

2.3. Data analysis and re-analysis of concentration-response data

Mean luminescence values of test chemicals and E2 standards were corrected for the response of the solvent controls. Resulting values were then divided by the maximum induction of the E2 standard (E2 max) to scale all values from 0 (solvent control) to 100% (E2 max). Average scaled values from triplicate experiments were plotted using the software GraphPad Prism 7.01 (GraphPad, San Diego, USA) and fitted using the Hill function with slope parameter 1 (Eq. (2)).

$$E(c_i) = \frac{\alpha_i \cdot c_i}{c_i + K_i} \tag{2}$$

where: $E(c_i)$ is the effect of a single chemical i at concentration c, α_i the efficacy (% E2 max), and K_i the concentration of i causing half-maximal effect.

Two approaches for determination of efficacy (α_i), which correspond to the maximum achievable effect of a single chemical i, were used to accommodate the requirements of the different modeling strategies. The concentration addition model, used in the EEF approach, requires that all curves are scaled from 0 (solvent control) to 1 (E2 max, 100%), while application of the GCA and IA models permitted use of substance-specific efficacies. For NP isomers investigated in the present study, these were not always defined by a clear upper asymptote, and were thus set to the mean value of measurements from the greatest concentration of the respective chemical.

2.4. Modeling of mixture effects

Effects of mixtures of NP isomers and co-exposures with E2 at 100 pM (appox. EC$_{50}$) or 1000 pM (approx. 100% effect), respectively, in the MVLN assay were predicted using three models: independent action (IA), concentration addition (CA) and generalized concentration addition (GCA).

Effects of mixtures of chemicals with various mechanisms of action can be described using the independent action (IA) model (Bliss, 1939; Eq. (3)). Since all investigated chemicals activate the ER, and thus,

share the same mechanism of action, this model is included in the present study only for comparison.

$$E_{mix} = 1 - \prod_{i=1}^{n} (1 - E(c_i)) \tag{3}$$

where: E_{mix} (0…1) is the relative effect of a mixture of n chemicals, and $E(c_i)$ (0…1) the effect of any single chemical i present in the mixture at concentration c.

The concentration addition (CA) model was first described by Loewe and Muischnek (1926) and can be used to describe the effects of mixtures of chemicals with the same mechanism of action. Assuming CA, each chemical can be expressed as the dilution of any other chemical in the mixture. It can be rearranged to result in the model described in Eq. (4) (Nweke et al., 2015.

$$E_{mix} = 100 - \frac{100}{1 + \sum_{i=1}^{n} \frac{c_i}{K_i}} \tag{4}$$

where: E_{mix} (%) is the relative effect of a mixture of n chemicals at concentration c, and K_i the concentration of chemical i causing half-maximal effect.

A generalized version of the concentration addition model (GCA) has been developed; its main advantage over the CA model is its ability to accurately predict effects of mixtures containing partial agonists, i.e. chemicals that have a lesser efficacy than other chemicals in the mixture (Howard et al., 2010; Eq. (5)). As a quantitative measure of model performance, the root mean squared error (RMSE) was calculated for each of the investigated models.

$$E_{mix} = \frac{\sum_{i=1}^{n} \frac{\alpha_i c_i}{K_i}}{1 + \sum_{i=1}^{n} \frac{c_i}{K_i}} \tag{5}$$

where: E_{mix} (%) is the effect of a mixture of n chemicals at concentration c, α_i the efficacy (% E2 max), and K_i the concentration of chemical i causing half-maximal effect.

2.5. Prediction of EEQs in environmental samples

Estradiol equivalent concentrations (EEQs) of 18 individual wastewater samples which were measured in the *E*-Screen assay were predicted from analytically determined concentrations of 11 natural and synthetic estrogens by means of the GCA model and the EEF approach (Spengler et al., 2001). This assay makes use of the ER-mediated proliferative effect of estrogens in the wild-type breast cancer cell line MCF-7 to quantify the concentration of natural and synthetic estrogens (Soto et al., 1995). Potencies and efficacies data of these 11 compounds have been previously published (Körner et al., 2001; Table 2). Concentrations less than the limit of detection were assumed to be negligible and set to zero (Table 3).

In the EEF approach (Eq. (1)) EEQs of wastewaters were calculated by use of the EEFs reported in the original publication (Körner et al., 2001; Eq. (1)). Furthermore, EEQs were calculated using the GCA model through a workflow (Fig. 2). Briefly, E_{mix} of the different wastewater samples was calculated using concentrations of individual putative ER agonists (Eq. (4)). This concentration, if tested in the *E*-Screen assay, could be referred to as a hypothetic concentration factor of 1 (L/L, i.e. the equivalent of 1 L of extracted sample per 1 L of cell culture media). Measured concentrations of the 11 chemicals were then multiplied by concentration factors spanning a range similar to a dilution series in the bioassay, and E_{mix} calculated to generate full concentration-response curves. These curves were fitted using Eq. (2) to determine EC$_{50}$s (LL^{-1}). The EC$_{50}$ of the E2 standard (ng L^{-1}) was divided by the EC$_{50}$ of the curve, resulting in an EEQ in ng L^{-1}. EEQs predicted using both approaches were compared to EEQs experimentally determined in the E-Screen assay (Körner et al., 2001). RMSEs were calculated as a quantitative measure of model performance.

I. Single chemical biotesting

1. For each compound that has been instrumentally quantified
- determine potency (*K*) and efficacy (% E2 max.)

II. Prediction of EEQs using GCA

1. Calculate the effect (% E2 max) of the mixture using GCA
- at the analytically determined concentrations of each chemicals

2. These concentrations can be re-scaled
- to a hypothetic concentration factor of 1 (L/L) if tested in an assay

3. Multiply the concentrations with varying concentration factors
- so that a full concentration-response curve can be established

4. Determine EC$_{50}$ (L/L) of the curve and calculate the EEQ (ng/L)
- through dividing the EC$_{50}$ of E2 (ng/L) by the EC$_{50}$ of the curve

III. Mass balance analysis

1. Test the environmental sample in the same assay as in I.
- at different concentration factors (L/L) to establish a full curve

2. Determine EC$_{50}$ (L/L) of the curve and calculate the EEQ (ng/L)
- through dividing the EC$_{50}$ of E2 (ng/L) by the EC$_{50}$ of the curve

3. Compare the predicted EEQs from II. with the measured EEQs from III. and calculate the percentage of unexplained effect

Fig. 2. Proposed workflow for predicting estradiol equivalent concentrations (EEQs) for complex mixtures and extracts of environmental samples using the generalized concentration addition (GCA) model.

3. Results and discussion

3.1. Re-evaluation of single chemical exposure data

Re-evaluation of the responses of MVLN cells to exposure with E2 and the NP isomers 4n–NP, *p*353-NP, *p*363-NP, *p*33-NP, *p*252-NP, *p*262-NP, and *p*22-NP that had been previously published by Preuss et al. (2006) led to differing best-fit values which accommodated demands of the various modeling strategies (Fig. 3). It should be noted that three of the investigated NP isomers (4n–NP, *p*262-NP, and *p*22-NP) did not cause measurable induction of ≥ 20% E2 max, which is often recommended as a stipulated threshold for meaningful effects in mass-balance analyses (Villeneuve et al., 2000). For this reason, no EC or EEF values were presented in the original publication (Preuss et al., 2006). In the present study, however, these data were included in the assessment to achieve better comparability between the different modeling approaches. Furthermore, responses of at least the highest tested concentration were significantly greater compared to the solvent control for the three chemicals (one-way ANOVA with Dunnett's post-hoc test, $p \leq 0.05$).

As required for using the best-fit data to model mixture effects according to CA, curves were extrapolated to 100% E2 max regardless of the maximum response achieved by the chemical. The obtained EC50 values were similar to the ones reported in the original publication (Fig. 3, Table 1; Preuss et al., 2006). When allowing for substance-specific efficacies while fitting the measured responses in the MVLN assay, fitted efficacies ranged from 6.70 to 63.2% E2 max (Table 1). This fitting strategy also resulted in markedly lesser estimates of potencies (for better differentiation termed *K* instead of EC50), which differed by a factor of 3 (*p*353-NP) to 33 (*p*262-NP).

3.2. Mixtures of E2 with individual NP isomers and mixtures of various NPs

Using descriptors of potency and efficacy of NP isomers described above, effects of co-exposures of single NP isomers with fixed concentrations of E2 (100 or 1000 pM) in the MVLN assay were predicted
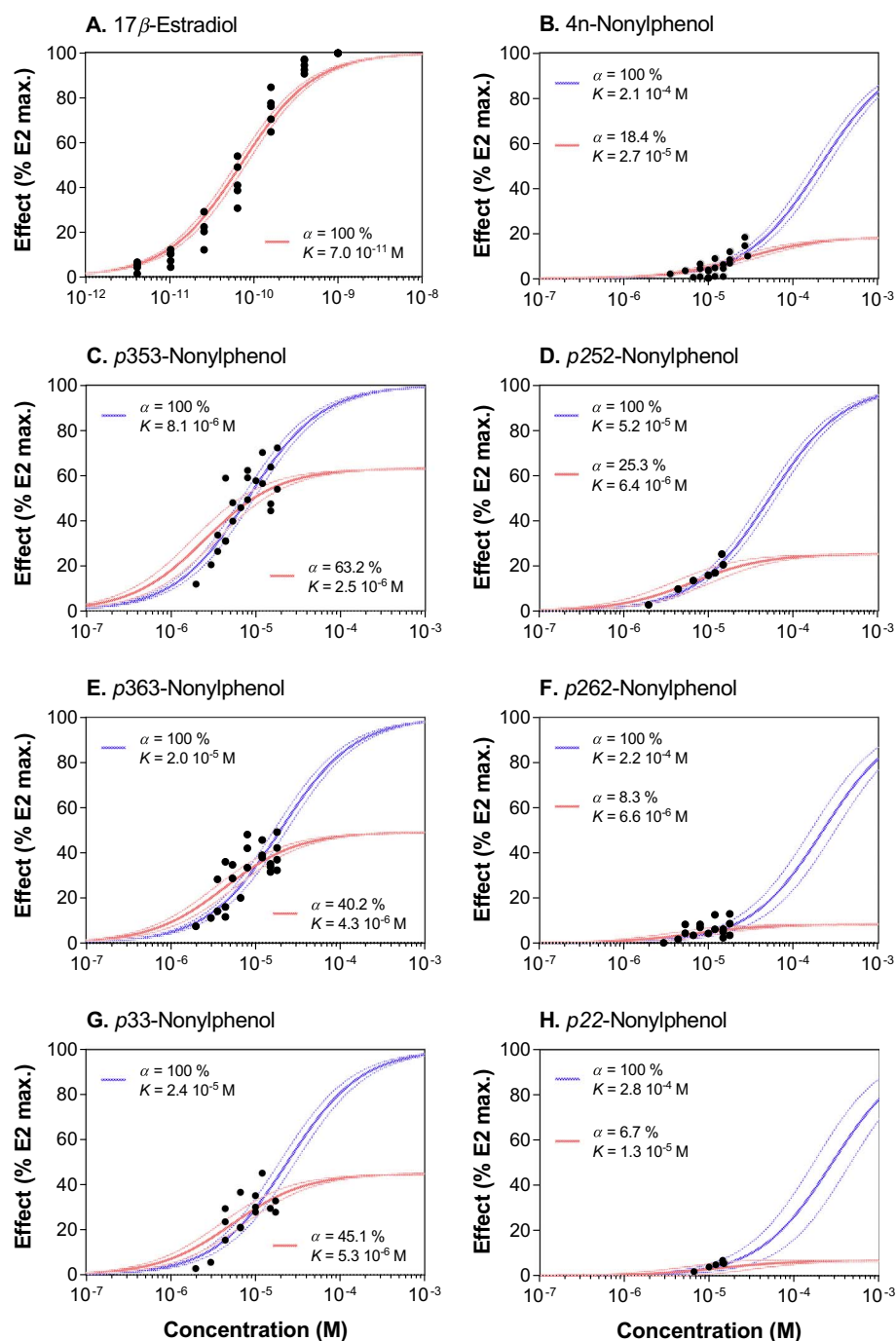
Fig. 3. Concentration-response curves of investigated nonylphenol isomers (NPs) and estradiol (E2) in the MVLN assay (closed circles) were measured in $n = 3$ independent biological replicates. Concentration values on the x-axis refer to nominal exposure concentrations. Dots represent mean values from triplicate technical measurements in one experiment. Experimental data were fitted using logistic regression, with bottom set to 0 and a slope value of 1. Red curves represent fits where the top value was set to the mean value of measurements in the highest concentration of the respective treatment group, while the top value was set to the maximum response of the E2 standard (100%) for fitting the blue curves. Raw data from Preuss et al. (2006) were re-analyzed for the purpose of this study. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

using the three models, and compared to the previously published experimental data (Fig. 4).

Predictions of the GCA model were generally most accurate (RMSE 13.0%), followed by CA and IA (RMSEs of 23.8 and 25.9%, respectively). As previously reported (Howard and Webster, 2009), the GCA model was the only model that was able to accurately describe the observed, competitive antagonism of partial agonists when mixed with full agonists, i.e. a decrease in the effect caused by E2 alone with increasing concentration of NP isomers. This phenomenon was observed for all isomers of NP with efficacies < 50% of maximum, regardless of whether they were mixed with 100 pM (approx. EC$_{50}$) or 1000 pM E2 (approx. 100% effect). With increasing concentrations of NP, the level of effect approached the efficacy of the NP isomer (Fig. 4). Only *p*353-NP with a value of 63.2% of maximum, exhibited an efficacy > 50%; for this isomer, co-exposure with 100 pM led to an increase in effect

exceeding that observed for E2 alone, while co-exposure with 1000 pM led to a decrease in effect caused by E2 alone with increasing concentration.

It was possible to demonstrate that GCA more accurately predicted behaviors of mixtures containing full and partial agonists of human ER in the MVLN reporter cell line. This observation supports previous findings that CA, which is also most commonly used to predict effects of mixtures of AhR agonists, failed to describe the often observed effect of competitive antagonism (Aarts et al., 1995; Harper et al., 1995; Howard et al., 2010; Safe, 1997). In a study by Howard et al. (2010), full agonists of the AhR (i.e. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or 2,3,7,8-tetrachlorodibenzofuran) were combined with either another full agonist (3,3′,4,4′,5-pentachlorobiphenyl), a partial agonist (2,3,3′,4,4′-pentachlorobiphenyl or galangin), or the competitive antagonist 3,3′-diindolylmethane. The authors found that both GCA and CA predicted

Table 1

Fitted estrogenic potencies K of the compounds investigated in the present study when applying different fitting strategies, e.g. efficacies α set to 100% E2 max (as needed for the EEF approach) and allowing for substance-specific efficacies α, respectively. All slopes were set to 1 and all lower asymptotes to 0%. Interpolated potency values K represent mean $\pm$ SEM determined from $n = 3$ independent biological replicates, with triplicate technical determinations per replicate.

Chemical	Efficacy α set to 100% E2 max		Efficacy α substance-specific		
	K (M)	R^2	K (M)	α (% E2 max)	R^2
E2	$7.0 \pm 0.6 \ 10^{-11}$	0.96	$7.0 \pm 0.6 \ 10^{-11}$	100	0.96
4n–NP	$2.1 \pm 0.2 \ 10^{-4}$	0.52	$2.7 \pm 0.5 \ 10^{-5}$	18.4	0.52
p353-NP	$8.1 \pm 0.8 \ 10^{-6}$	0.60	$2.5 \pm 0.5 \ 10^{-6}$	63.2	0.56
p363-NP	$2.0 \pm 0.2 \ 10^{-5}$	0.36	$4.3 \pm 0.7 \ 10^{-6}$	40.2	0.55
p33-NP	$2.4 \pm 0.3 \ 10^{-5}$	0.40	$5.3 \pm 1.0 \ 10^{-6}$	45.1	0.57
p252-NP	$5.2 \pm 0.5 \ 10^{-5}$	0.88	$6.4 \pm 1.9 \ 10^{-6}$	25.3	0.71
p262-NP	$2.2 \pm 0.4 \ 10^{-4}$	0.17	$6.6 \pm 3.0 \ 10^{-6}$	8.30	0.23
p22-NP	$2.8 \pm 0.8 \ 10^{-4}$	0.55	$1.3 \pm 0.9 \ 10^{-5}$	6.70	0.41

Table 2

Estrogenic potencies K (M), efficacies α (% E2 max), and estradiol equivalence factors (EEFs) for a range of natural and synthetic estrogens in the *E*-Screen assay. Table modified from Körner et al. (2001). The authors of the original publication did not report estimates of biological variability.

Chemical	K (M)	α (% E2 max)	EEF ($-$)
Estrone	$6.7 \ 10^{-11}$	112	$9.6 \ 10^{-2}$
17β–Estradiol	$6.1 \ 10^{-12}$	100	$1.0 \ 10^{0}$
17α–Ethinylestradiol	$5.2 \ 10^{-12}$	105	$9.1 \ 10^{-1}$
Mestranol	$2.0 \ 10^{-10}$	100	$1.3 \ 10^{-2}$
Genistein	$1.8 \ 10^{-8}$	123	$2.8 \ 10^{-4}$
α-Endosulfane	$2.0 \ 10^{-7}$	70.0	$4.8 \ 10^{-6}$
Di-*n*-butylphthalate	$2.5 \ 10^{-5}$	63.0	$3.4 \ 10^{-7}$
Benzyl-*n*-butylphthalate	$3.1 \ 10^{-6}$	80.0	$2.4 \ 10^{-6}$
Bisphenol A	$1.5 \ 10^{-7}$	97.0	$5.3 \ 10^{-5}$
4-Nonylphenol (techn.)	$9.9 \ 10^{-8}$	105	$7.6 \ 10^{-5}$
4-nonylphenoxyacetic acid	$7.3 \ 10^{-7}$	54.0	$1.4 \ 10^{-5}$

the effects of a mixture of full agonists equally well, while in all other cases the GCA model outperformed CA. The difference between the two modeling approaches was directly dependent on the efficacy of the

partial agonist. A similar trend was observed in the present study, where RMSEs for the predicted mixture effects of co-exposures with E2 (Fig. 4) increased more drastically with decreasing efficacy for the CA model compared to the GCA model (data not shown).

Another aspect that had been observed previously is that GCA was also capable of predicting effects of pure antagonists: the effect of the AhR antagonist 3,3′-diindolylmethane, a compound that binds the receptor without activating it, was accurately predicted by GCA (Howard et al., 2010). In this context, GCA might also have the potential to help understanding the effects of pure antagonists of the ER which are used as human pharmaceuticals (e.g. diethylstilbestrol, fulvestrant, and tamoxifen) and their interaction with endogenous estrogens (Riggs and Hartmann, 2003). GCA could thus also be useful in pharmacological research, for targeting drugs and predicting effects of potential co-medication.

When toxicity of ternary and quaternary mixtures of various NP isomers was assessed with the three models, CA and IA again significantly overestimated effects of mixtures, with RMSEs of 45.8 and 51.0%, respectively (Fig. 5). Predictions of the GCA model were most accurate, with a RMSE of only 15.7%. As in all tested serial dilution levels mixture ratios were constant, this experiment can be considered to reflect the situation of testing complex mixtures, e.g. extracts of environmental samples. The results of these experiments suggest that similarly greater predictive power of the GCA approach could also be expected for extracts of environmental samples.

3.3. Prediction of EEQs in environmental samples

It has long been recognized that the testing of extracts of environmental samples using bioanalytical in vitro assays and the use of that data in potency-balance analyses, results in a dilemma: the CA model, or more specifically the EEF/TEF approach, requires that the curves used in the analysis show equal efficacy and parallelism of slopes. These requirements are unrealistic for most mixtures and are rarely met by data for individual agonists of the ER or AhR and in fact without knowledge of the concentrations of agonists in extracts of environmental samples cannot be verified (Giesy and Kannan, 1998). Thus, it has been proposed and widely accepted that relative potency estimates for single chemicals and extracts of environmental samples should better be expressed as multiple point estimates (e.g. $EC_{20} – EC_{80}$ ranges) (Villeneuve et al., 2000) or even probability functions (Putzrath, 1997).

Table 3

Estradiol equivalent concentrations (EEQs) for 18 wastewater samples were predicted from analytically determined concentrations of 11 natural and synthetic estrogens by means of the GCA model and the EEF approach, and compared to EEQs determined in the *E*-Screen assay. Analytical data, as well as measured EEQs were taken from the literature (Körner et al., 2001; Spengler et al., 2001). Concentrations less than the detection limit were assumed to be zero.

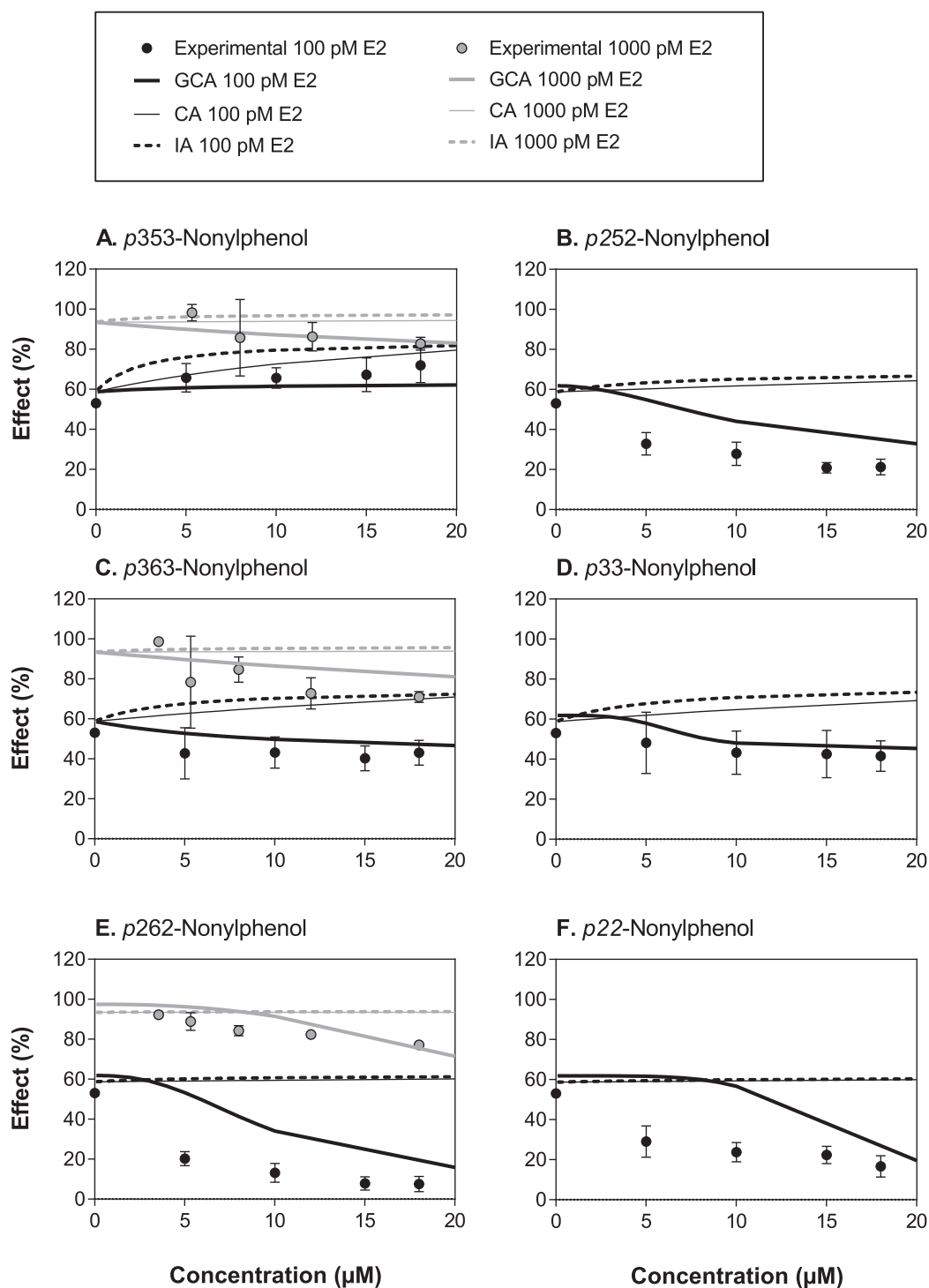| Sample	Measured	Predicted (GCA)		Predicted (EEF)	
	EEQ (ng L^{-1})	EEQ (ng L^{-1})	% difference	EEQ (ng L^{-1})	% difference
Ditzingen	3.20	4.32	35	5.98	87
Ludwigsburg-Eglosheim	0.94	0.14	85	0.05	95
Ludwigsburg-Poppenweiler	1.10	0.17	85	0.07	94
Stuttgart-Mühlhausen	3.30	2.34	29	2.66	19
Stuttgart-Möhringen	3.20	6.66	108	10.23	220
Stuttgart-Büsnau	1.30	3.93	202	4.75	265
Hechingen	1.20	8.66	622	17.21	1334
Albstadt-Ebingen	0.21	0.22	5	0.06	71
Sindelfingen	7.80	9.60	23	17.55	125
Donaueschingen	2.40	3.60	50	4.83	101
Blaubeuren	1.40	4.17	198	5.43	288
Ulm	3.30	2.35	29	2.35	29
Pforzheim	1.20	2.93	144	3.19	166
Industrial treatment plant 1	1.80	0.29	84	0.09	95
Industrial treatment plant 2	2.70	4.62	71	7.14	164
Lahr	1.10	3.13	185	3.65	232
Waiblingen	NQ	1.99	–	2.22	–
Leutkirch	0.65	1.02	57	0.80	23

NQ: not quantifiable.

Fig. 4. Concentration – response curves of MVLN cells co-exposed to mixtures of six nonylphenol isomers with either 100 pM (black) or 1000 pM E2 (gray). Symbols represent the mean ± standard deviation n = 2–3 independent biological replicates, with triplicate technical replicates each. Solid lines represent predictions of the GCA model, thin lines of the CA model, and dashed lines of the IA model. Raw data from Preuss et al. (2010) were re-analyzed for the purpose of this study.

Data requirements for using GCA to predict the potency of extracts of environmental samples are equivalent to the EEF approach: Concentration-response curves for each constituent of the mixture need to be established anyways; the only additional requirement is that a different curve-fitting strategy needs to be applied in order to allow for substance-specific efficacies. To predict EEQs in extracts of environmental samples by means of the GCA approach, we propose a simple methodology using this data (Fig. 2). Briefly, for each chemical that has been determined analytically, EC_{50} and efficacy values need to be

compiled or generated. Then, the effect of the mixture at the analytically determined concentration of each chemical is calculated, representing a hypothetic concentration factor of 1 L/L (i.e. the equivalent of 1 L of extracted sample per 1 L of cell culture media) in the applied bioassay. Next, the effects of the mixture at varying concentration factors are predicted, emulating a dilution series in a bioassay experiment. To this end, measured concentrations of individual constituents of the mixture are multiplied by a range of concentration factors, and the effect of the mixture predicted using GCA.
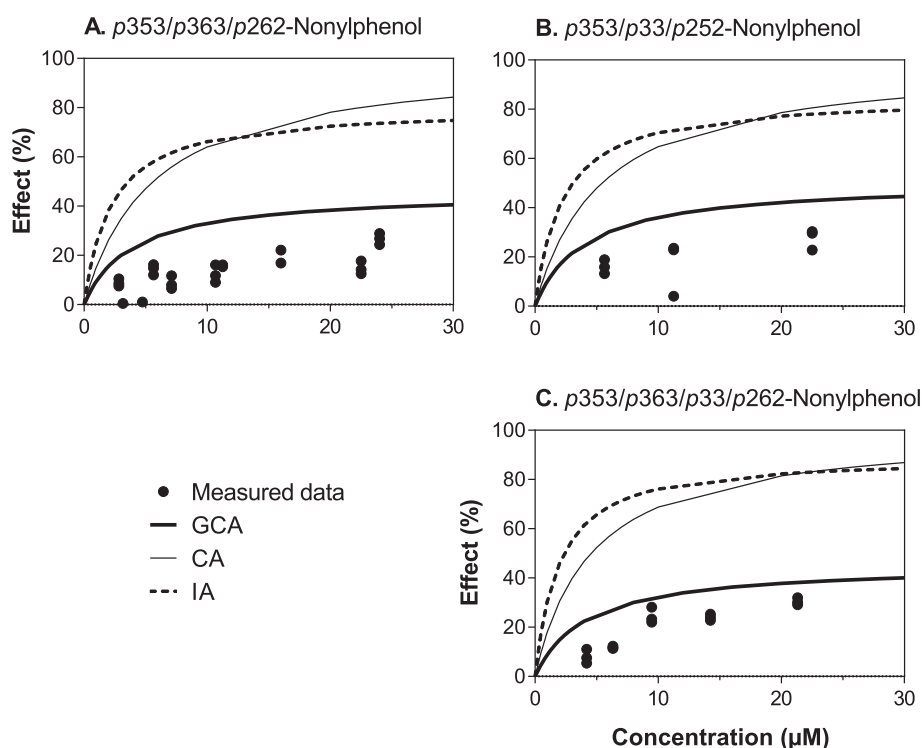
Fig. 5. Concentration – response curves of MVLN cells exposed to mixtures of three to four nonylphenol isomers at fixed concentration ratios. Symbols represent the mean values from $n = 3$ independent biological replicates, with triplicate technical replicates each. Solid lines represent predictions of the GCA model, thin lines of the CA model, and dashed lines of the IA model.

From these effect values, a concentration-response curve of the mixture can be established, and the EC_{50} determined. Predicted EEQ of samples can then be calculated by dividing the measured EC_{50} of the E2 standard by the predicted EC_{50} of the curve. These predicted EEQs can then be compared with experimentally determined EEQs for the same sample, and mass-balance analyses conducted to determine the fraction of measured effect explained by the analytically quantified chemicals.

To test the accuracy of this approach, a previously published dataset of concentrations of various steroidal and non-steroidal xenoestrogens in sewage treatment effluents was analyzed. Effects of extracts of these samples were determined in the *E*-Screen assay (Körner et al., 2001; Spengler et al., 2001). When EEQs of these samples were predicted using the GCA approach for environmental samples (Fig. 2), the CA model/EEF approach significantly overestimated potencies of samples, when compared to EEQs estimated by use of bio-analytical techniques (RMSE of 5.3 ng L^{-1}), while predictions of GCA were twice as accurate, with a RMSE of 2.5 ng L^{-1} (Fig. 6). In conclusion, the GCA model provides a powerful alternative to the established EEF approach, while requiring the same amount of experimental data for parameterization. While the EEF approach can still be considered an appropriate first-tier model, the resulting lower proportion of unknown effect potential when using the GCA model will significantly streamline the process of identifying previously undescribed xenoestrogens. As this study addressed a limited chemical space and only one sample matrix, i.e. wastewater, future dedicated research will need to show whether this approach will also be useful for analysis of data from other bioassays which focus on other nuclear receptors, e.g. the AhR, or completely different modes of action (e.g. mutagenicity, genotoxicity, oxidative stress response), and different sample matrices, e.g. sediments, blood plasma, or tissue extracts. Furthermore, it will need to be shown if the model could also be applied to concentration-response relationships other than the four-parameter Hill curve.

4. Conclusions

In this study, we could show that the GCA model was able to consistently make more accurate predictions of the potency of mixtures and
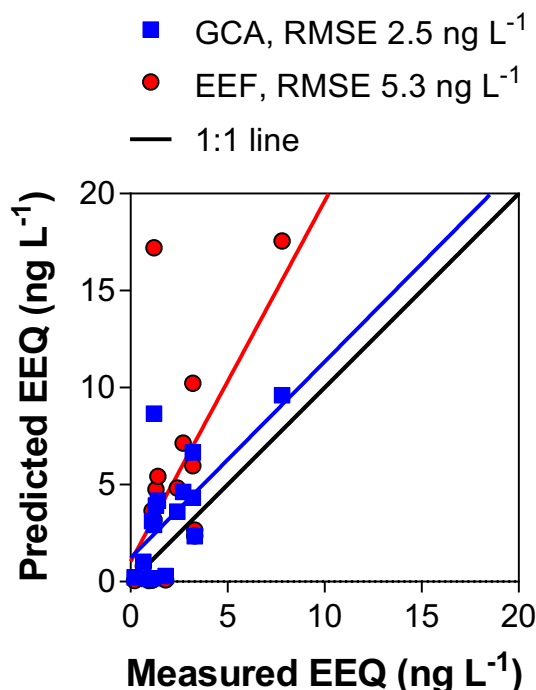

Fig. 6. Comparison of estradiol equivalent concentrations (EEQs) of wastewater samples in the E-Screen assay, with EEQs predicted using generalized concentration addition (GCA, Fig. 2) and the estradiol equivalence factor (EEF) approach from analytically determined concentrations of 11 natural and synthetic estrogens. Each symbol corresponds to one individual wastewater sample. The root mean squared error (RMSE) of the prediction is provided in the legend.

extracts of environmental samples determined using in vitro assays compared to the classical EEF approach if the samples contained partial agonists. These findings are of great relevance to mass balance analyses, and thus highly relevant to risk assessments. Being able to more accurately estimate the fraction of estrogenicity that remains unexplained

by chemical analyses is vital to trigger dedicated efforts to identify the causative chemicals. Nuclear receptors other than the ER and AhR increasingly gain importance when dealing with the effects of CECs. Using GCA and thereby accounting not only for agonists of these receptors, but also a plethora of partial agonists and antagonists in the environment, will lead to a better understanding of the mixture effects of a vast number of other environmental contaminants of concern.

Transparency document

The http://dx.doi.org/10.1016/j.tiv.2017.10.022 associated with this article can be found, in online version.

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