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Assessing ecotoxicity and the endocrine potential of selected phthalates, BADGE and BFDGE derivatives in relation to environmentally detectable levels

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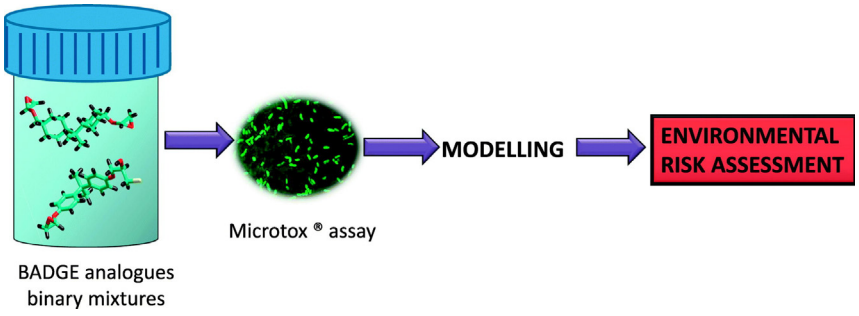
HIGHLIGHTS

- The Concentration Addition model enables more reliable threat detection of BADGE and its analogues in binary mixtures.
- BADGE analogues may pose an equal threat to ecosystems as the parent com-pound itself.
- Numerous cases of synergetic and a few antagonistic effects of BADGE analogues were observed.
- Most of the binary mixtures reveal independent mode of action when present in binary mixtures.

GRAPHICAL ABSTRACT



abstract

There is no doubt that the subject area of plastic materials (e.g., production of epoxy resins or polyesters) is inherently connected to issues concerning bisphenol A (BPA) and its analogues. Unfortunately, much less attention has been given to other compounds, which are also used for the production of these materials. Bisphenol A diglycidyl ether (BADGE) is a synthetic industrial compound obtained by a condensation reaction between epichlorohydrin (ECH) and BPA. Similarly, novolac glycidyl ether (BFDGE) is produced in the reaction between novolac and epichlorohydrin. Nevertheless, there is a lack of information on the combined effects of BADGE derivatives at environmentally relevant levels. In the current study, toxicity levels in Microtox® and XenoScreen YES/YAS assays were determined for several analogues alone, then the biological effects of compound pairs mixed in 33, 66 and 100% of each compounds' EC_{50} ratios were evaluated. The Microtox® test has been chosen as a relevant tool, and the results were referred to the Xenoscreen YES/YAS assay, which has been chosen for the fast determination of the endocrine potential of the com-pounds tested. The results obtained constitutes the basis for model studies, with Concentration Addition (CA) and Independent Action (IA), followed by Model Deviation Ratio (MDR) interpretation, to evaluate the possible interac-tions occurring between analytes when present in mixtures. The results indicate that the hydrochloric derivatives of BADGE and BFDGE are of the greatest toxicological and endocrine threat. Thus, their presence in mixtures under cer-tain environmental conditions (including presence in the tissues of living organisms) should be strictly monitored and reported, especially in acidic environments. Strong evidence on the synergic behaviors of these analytes, which expressed high toxicity (EC_{50} 2.69–117.49 μg/mL), is demonstrated with Model Deviation Ratio (MDR).

Keywords: Plasticizers, Phthalates, Bisphenol A diglycidyl ether, derivatives Binary mixtures toxicity, modelling Microtox®, Xenoscreen YES/YAS, Model deviation ratio (MDR)

Abbreviations: CA, Concentration Addition; IA, Independent Action; ECH, epichlorohydrin; BPA, bisphenol A; BADGE, bisphenol diglycidyl ether; BFDGE, bisphenol F diglycidyl; BPA, bisphenol A; EFSA, European Food Safety Authority; EU, European Union; LOEC, Lowest Observable Effect Concentration; MDR, Model Deviation Ratio; NOEC, No-Observed Effect Concentration; TDI, Tolerable Daily Intake; SML, Specific Migration Limit.

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1. Introduction

The development of the chemical industry, which ensures the possibility of creating a broad range of materials that are durable, light, cheap and easy to process and produce, has undoubtedly contributed to the development of civilization and the improvement of the standard of living in society in recent years. Plastics are now the most universal and multi-functional materials used in each field of technology and industry (Al-Natsheh et al., 2015). Despite the numerous advantages of these materials there are some concerns that chemical constituents of plastics may have some harmful effects on wildlife and environment (i.e. air, land, water, groundwater pollution, harmful to biota) (Bakir et al., 2012; Rochman et al., 2013; Thompson et al., 2009).

Much of the subject area of plastic materials is inherently connected with issues concerning bisphenol A (BPA). For many years, research has been conducted on its environmental fate, as well as its influence on living organisms. Unfortunately, much less attention has been given to other compounds, which are also used for the production of those plastic materials. Bisphenol A diglycidyl ether (BADGE) is a synthetic industrial compound obtained by a condensation reaction between epichlorohydrin (ECH) and BPA (Xue et al., 2015). Similarly, novolac glycidyl ether (BFDGE), a complex compound with more than two aromatic rings and glycidyl groups, is produced from the reaction between novolac and epichlorohydrin. Both BADGE and BFDGE are used mainly for the production of epoxy resins but also as an additive for polyesters and a hydrogen chloride binding agent during varnished surface degradation (Grob et al., 2010). Many industrial branches use these because of the excellent properties for epoxy resins, including chemical and heat resistance, good mechanical properties and very good electrical insulating properties. Epoxy resins are used as adhesives, food-can inner coatings for protecting food from metal contamination and preventing metal corrosion, components of powder coatings in polymer-based root canal sealers and in electrical systems and electronics (Chang et al., 2014; Xue et al., 2015). In Table 1, key information on the compounds used in this research is summarized.

Unfortunately, despite the numerous pros resulting in its common industrial use, epoxy resins may also be a source of contamination. As indicated in numerous research results, these compounds may be washed off the material surface and transferred to food or individual elements of the environment due to the interaction with food ingredients or the influence of external factors (Ballesteros-Gómez et al., 2007; Pérez-Palacios et al., 2012). The identification and the quantitative determination of the released compounds is a particularly difficult task, due to the presence of the highly reactive, easily transformed epoxide moieties in its structure. In aqueous environments, BADGE and BFDGE form water-soluble products, such as BADGE·$2H_2O$, BADGE·H_2O and BFDGE·$2H_2O$, BFDGE·H_2O, respectively.

Reaction with hydrochloric acid yields BADGE·HCl·H_2O, BADGE·2HCl, BADGE·HCl, BFDGE·2HCl and BFDGE·HCl (Petersen et al., 2008; Xue and Kannan, 2016). For most compounds, a toxicological analysis was conducted, which demonstrated that they induce adverse effects and show hormonal activity in living organisms (Satoh et al., 2004; Sueiro et al., 2006, 2003). Moreover, many of this xenobiotics are characterised by lipophilicity thereby they are able to easily pass through biological membranes and penetrate living cells, thus be subject to bioaccumulation in various kinds of tissue and organs. Mass production combined with its physiochemical properties results in a significant contribution of those substances to the general negative environmental impact (Wang et al., 2015; Xue et al., 2016). Some of physicochemical properties and annual global production are summarized in Table 1. That is why it is necessary to intensify the work of determining the exact levels of xenobiotics in individual elements of the environment, as well as the exact level of their influence on living organisms.

1.1. Legal aspects of BADGEs and BFDGEs derivatives and phthalates applications

In 2005, the European Food Safety Authority (EFSA) concluded that BADGE does not raise concern for carcinogenicity and genotoxicity, according to in vivo studies. On the basis of the NOAEL (No-Observed-Adverse-Effect Level defined as a highest dose at which there was not an observed toxic and adverse effect) value of 15 mg/kg bw/day, the tolerable daily intake (TDI) value was determined to be 0.15 mg/kg/bw for BADGE (Møller et al., 2012). For other related compounds, TDI values have, so far, not been determined. Considering that other compounds are suspected of showing similar or even higher endocrine and toxic effects than BADGE, it seems necessary to update the existing legal regulations. As was previously mentioned, BADGE and related compounds are used mainly for the production of the inner coatings of packaging materials. To ensure maximum consumer safety, the European Commission, by virtue of the Commission Regulation no. 10/2011, has established Specific Migration Limits (SLM) for individual compounds. The EU specified a maximum SML of 9 mg/kg for foodstuff or simulated liquid for the sum of BADGE and its hydrolytic products and 1 mg/kg for chlorinated derivatives. The use of BFDGE was prohibited in food contact applications in 2005 in the EU (Commision Regulation no. 2011/8/EU). Moreover some of compounds used to production of plastic materials are included in the scope of the European Chemicals Regulation (No 1907/2006 on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)) (Geueke and Muncke, 2017) Between 2008 and 2014 four of phthalates (Diisobutyl phthalate, Dibutyl phthalate, Benzyl butyl phthalate and Bis(2-ethylhexyl) phthalate), two primary aromatic amines (4,4′-Methylenebis[2-chloroaniline], and 4,4′-Methylenedianiline), and three halogenated compounds (Trichloroethylene, Tris(2-chloroethyl)phosphate and Hexabromocyclododecane) was added to Annex XIV of REACH (Geueke and Muncke, 2017, European Parliament, 2011, Commision Regulation (EU) No 125/2012 of 14 February 2012, Commision Regulation (EU) No 348/2013 of 17 April 2013).

1.2. Analytical aspects of BADGE and related compounds determination

An appropriate approach of enabling the isolation and enrichment of analytes, as well as the choice of an appropriate final determination technique, is necessary to obtain reliable analytical information. This information is needed for the assessment of the condition of individual elements in the environment, as well as the human risk. Literature reviews unambiguously show that the main topics of interest of chemical analysts is the identification and determination of BADGE derivatives released from the surface of packaging materials. Those compounds are determined in both samples of packaging materials themselves and samples of simulant liquids and food (Cunha et al., 2012; Gallo et al., 2017; Lapviboonsuk et al., 2014; Suciu et al., 2013). Environmental samples and biological material have been of interest in the context of the presence of these analytes for only a few years. The use of modern analytical techniques has provided information on the levels of most BADGE derivatives in samples of inside air, dust and biosolids (Tran et al., 2016; Xue et al., 2016, 2015). BADGE, BFDGE and their derivatives have also been identified in plasma and adipose samples. More information on the content of BADGE-related compounds in various types of samples is summarized in Table 2.

1.3. Biological methods in studies on BADGEs

Currently in modern analytical chemistry, bioanalytical methods are becoming more important for the assessment of the condition of the environment (Wieczerzak et al., 2016a). The main asset resulting from the use of living organisms in this kind of research is the ability to assess the combined activity of all the contaminants present in the sample, as well as the types of interactions and modes of action of chemical compounds.

In the literature, an abundance of information may be found on both the toxic and endocrine disrupting activity of most compounds. The previous research has demonstrated that BADGE and its reaction products have shown estrogenic activity and androgen antagonist activity (Gallart-Ayala et al., 2011; Satoh et al., 2004). Information on biological tests used to assess the toxic effect of BADGE derivatives, as well as the mixture of compounds released from the surface of packaging materials is compiled in Table 3.

In light of the current toxicological knowledge and the results of studies aimed at the best possible understanding of toxic interactions between contaminations, it is known that the coexistence of a few compounds at very low levels of concentration may result in a toxic effect. The joint activity of a few toxic substances may contribute to the reinforcement of the observed adverse effect (i.e., synergy) or its weakening (i.e., antagonism) (Li et al., 2017; Ramirez et al., 2014). This phenomenon has become a stimulating factor for research aiming at estimating the overall impact of mixtures of contaminants. The effects of individual and combined toxicity of bisphenol A, dibutyl phthalate and cadmium on oxidative stress and genotoxicity were assessed using HepG2 cells. Other research has focused on determining the overall impact of a mixture of di (2-ethylhexyl) phthalate (DEHP) and polychlorinated biphenyls (PCBs) on reproductive dysfunction in the adult male mouse. It was determined that the mixture induced permanent alterations in reproductive health in a different way from the single compounds (Fiandanese et al., 2016). In other studies, the antagonistic and synergic effect between nonylphenol and di-N-butyl phthalate was observed (Hu et al., 2014). According to our current knowledge, there is no information concerning the assessment of the overall activity of mixtures of BADGE-related compounds. The main objective of the research was to assess the dependencies which occur between compounds released from the surface of epoxy resin.

2. Experimental and methodology

Experimental design is presented in more detail in (Wieczerzak et al., 2016b); below, we present the basic information on the chemicals used and methodologies applied during the studies (refer to Fig. 1. to see general scheme of the methodology used).

2.1. Chemicals and reagents

Model substances selected for the study, namely: BADGE (CAS 1675-54-3), BADGE·H_2O (CAS 6002-91-0), BADGE·$2H_2O$ (CAS 5581-32-8), BADGE·HCl (CAS 13836-48-1), BADGE·2HCl (CAS 4809-35-2), BADGE·H_2O·HCl (CAS 227947-06-0), BFDGE (CAS 2095-03-6), BFDGE·$2H_2O$ (CAS 72406-26-9), BFDGE·2HCl (CAS 374772-79-9), DEP (CAS 84-66-2), DBP (CAS 84-74-2), DEHP (CAS 117-81-7), NOGE (CAS 158163-01-0) and BIS-DMA (CAS 3253-39-2) were purchased from Sigma Aldrich (Germany). It holds true also for other chemicals used for research, namely: HPLC grade methanol (CAS 67-56-1), dimethyl sulfoxide (DMSO). Ultra-pure water was obtained using grade A10 Milli-Q system (Millipore). Standard stock solution of each compound was prepared separately by dissolving respective standard (to reach the concentration of 4 mg/mL) in HPLC grade methanol and stored in −20 °C. Various working solutions were obtained by serial dilution of the stock solutions with HPLC-grade methanol or ultrapure Milli-Q water. The concentration ranges [μM] for analytes studied in order to determine their EC_{50} respective data and subsequently to select C_1, C_2 and C_3 (being 33, 66 and 100% of EC_{50} of respective analyte) and determine impact of their co-presence on toxicity levels are listed in Table 4. (together with LOEC (Lowest Observable Effect Concentration) and NOEC (No-Observed Effect Concentration) of given chemicals with respect to Xenoscreen YES/YAS).

2.2. Microtox® reagents and methodology

The Microtox® test acute reagent (lyophilized *Vibrio fischeri*), osmotic adjustment solution (OAS, 22% solution of sodium chloride), reconstitution solution (RS), and diluent (2% solution of sodium chloride) were purchased from Modern Water (USA). The study was conducted using Microtox® analyzer model 500 (USA). Apparatus is equipped with 30 incubation wells as well as reagent (bacterial suspensions) and read wells. Temperatures are assigned to the corresponding type of performed test (in this case acute toxicity test) and internally maintained at 5.5 ± 1.0 °C for reagent well and 15.0 ± 0.5 °C for both the incubator part and the read well. pH was adjusted to fall within the 6.5–7.5 range with NaOH (CAS no. 1310-73-2) and HCl (CAS no. 7647-01-0) (purchased from Avantor Performance Materials S.A. (Poland)) using Metrohm pH-meter model 827 (Poland).

The EC_{50} parameter for each analyte of interest separately was determined by standard protocol using the Microtox® Analyzer Model 500 and serial dilutions. Lyophilized reagent with *Vibrio fischeri* bacteria was hydrated with 1 mL of RS and maintained at 5.5 ± 1.0 °C, subsequently 100 μL of bacterial solution and a pre-made samples of standard dissolved in distilled water (made from stock solutions of given analyte dissolved in ethanol) were added into the vials. To produce a suitable osmotic pressure (above 2%) OAS was added to the vial with the highest concentration and proper dilutions and ions additions were prepared. The incubation time was 30 min. Range-screening test for insoluble substance was also performed to narrow the range of concentrations tested, afterwards proper tests were performed in triplicates to determine range of linearity and calculate particular analytes EC_{50} values.

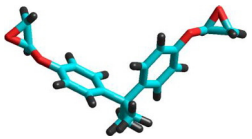
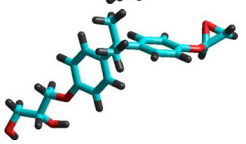
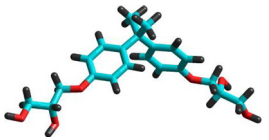
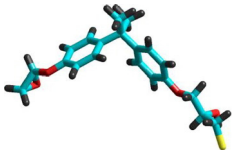
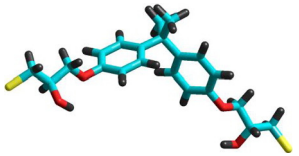
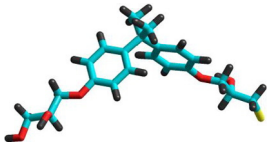
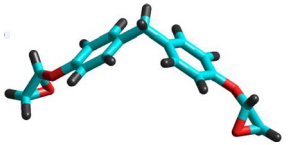
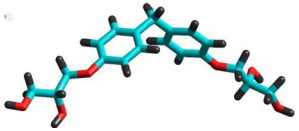
In order to determine whether the addition of one analyte to solution of another one would change the bioluminescence of bacterial suspension, concentrated solutions of the compounds were prepared. Test mixtures were prepared in such a way that the compounds were present in an appropriate ratio respectively 100% of first model substance and the second substance with a reduced effect to 33% and 66% of EC_{50}. Incubation time of samples with bacteria for all of the tests was 30 min.

2.3. Xenoscreen YES/YAS reagents and methodology

A set of XenoScreen YES/YAS was purchased from Xenometrix AG (Switzerland), namely: vial with hERα yeasts (to determine estrogenic activity) and hAR (to determine androgenic activity) settled on the filtration paper, basal medium, vitamin solution, L-aspartic acid solution, L-treonine solution, $CuSO_4$, 17β-estradiol (E2, YES+ control), 5α-dihydrotestosterone (DHT, YAS+ control), 4-hydroksytamoxyphene (HT, YES- control), flutamide (FL, YAS- control), DMSO. CPRG (Chlorophenol Red-β-D-galactopyranoside) was purchased from Sigma Aldrich (Germany). Measurement of cell density (wavelength 690 nm) and of the intensity of the CPRG transformation product (wavelength 570 nm) was performed with a TECAN Infinite M200 spectrophotometer.

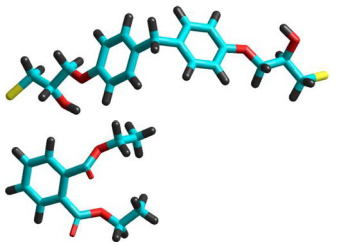
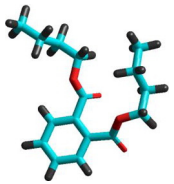
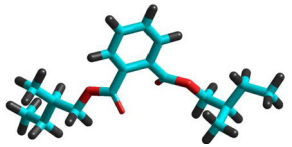
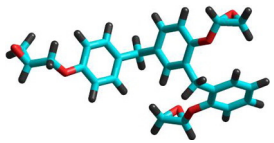
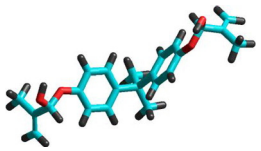
To investigate endocrine potential of selected compounds slightly modified protocol of XenoScreen YES/YAS was utilized, which uses genetically modified yeast cells of *Saccharomyces cerevisiae*. For this purpose the DNA sequence of human oestrogen hERα or androgen hAR receptors was stably integrated into the main chromosome of the yeast cells. Yeasts exposed to compounds that have endocrine potential produce β-galactosidase, which oxidizes the dye CPRG in growth medium. The interpretation occurs by measuring the density of the cell suspension and the color saturation of the oxidized dye. Furthermore, the cells also contain an expression plasmid carrying the lacZ reporter gene encoding the enzyme β-galactosidase and means responsive to estrogens (YES) or androgen (YAS). The yeast cells were cultured from the filter papers in growth medium (basic medium with a vitamin solution, solution of L-threonine, L-aspartic acid and copper (II) sulfate (VI)).

Table 1
Basic information on compounds used in the research.

Compound (IUPAC name)	Acronym	CAS no.	Molecular weight [g/mol]	K_{ow}	pKa	Water solubility at 25 °C [mg/L]	TDI [mg/kg]	Annual production (year)	Optimized structure[1]	Distances [Å]			Angle [°]
										O-C[2]	O-O[3]	C-O[4]	O-C-O[5]
2-[[4-[2-[4-(Oxiran-2-ylmethoxy)phenyl]propan-2yl]phenoxy] methyl]oxirane	BADGE	1675-54-3	340,41	3,84	–	3,7	0,15	31 k tonnes (2003)		5,78	9,28	5,78	108,83
3-[4-[2-[4-(Oxiran-2-ylmethoxy)phenyl]propan-2-yl]phenoxy]propane-1,2-diol	BADGE $\cdot H_2O$	76,002-91-0	358,43	3,09	13,53	12,6	0,15	–		5,76	9,28	5,76	107,78
3-[4-[2-[4-(2,3-Dihydroxypropoxy)phenyl]propan-2-yl]phenoxy]propane-1,2-diol	BADGE $\cdot 2H_2O$	5581-32-8	376,44	1,93	13,23	96,2	0,15	–		5,72	9,31	5,73	105,41
1-Chloro-3-[4-[2-[4-(oxiran-2-ylmethoxy)phenyl]propan-2-yl]phenoxy]propan-2-ol	BADGE·HCl	13,836-48-1	376,87	4025	13,33	5,25	–	–		5,76	9,17	5,76	105,57
1-Chloro-3-[4-[2-[4-(3-chloro-2-hydroxypropoxy)phenyl] propan-2-yl]phenoxy]propan-2-ol	BADGE $\cdot 2HCl$	4809-35-2	413,33	4340	12,83	0,32	–	–		5,75	9,28	5,74	107,74
3-[4-[2-[4-(3-Chloro-2-hydroxypropoxy)phenyl]propan-2-yl]phenoxy]propane-1,2-diol	BADGE $\cdot H_2O \cdot HCl$	227,947-06-0	394,89	3250	–	5,5	–	–		5,76	9,15	5,76	105,11
2-[[4-[[4-(Oxiran-2-ylmethoxy) phenyl]methyl]phenoxy] methyl] oxirane	BFDGE	2095-03-6	312,37	3,26	–	17,2	–	–		5,71	9,50	5,71	112,53
3-[4-[[4-(2,3-Dihydroxypropoxy)phenyl]methyl] phenoxy]propane-1,2-diol	BFDGE $\cdot 2H_2O$	72,406-26-9	348,39	1254	13,23	453	–	–		5,70	9,42	5,70	111,44
2-Chloro-3-[4-[[4-(2-chloro-3-hydroxypropoxy)phenyl] methyl]phenoxy]propan-1-ol	BFDGE $\cdot 2HCl$	374,772-79-9	385,28	3,98	13,52	1,51	–	–		5,71	9,52	5,71	112,85

(continued on next page)

Table 1 (*continued*)

Compound (IUPAC name)	Acronym	CAS no.	Molecular weight [g/mol]	K_{ow}	pKa	Water solubility at 25 °C [mg/L]	TDI [mg/kg]	Annual production (year)	Optimized structure[1]	Distances [Å]			Angle [°]
										O-C[2]	O-O[3]	C-O[4]	O-C-O[5]
Diethyl benzene-1,2-dicarboxylate	DEP	84-66-2	222,24	2470	–	1080	5	4,5 k tonnes (2008)		2,45	3,79	2,46	74,44/85,21
Dibuthyl benzene-1,2-dicarboxylate	DBP	84-74-2	278,34	4500	–	11,2	0,01	26 k tonnes (1998)		2,46	3,95	2,45	65,16/89,62
Bis(2-ethylhexyl) benzene-1,2-dicarboxylate	DEHP	117-81-7	390,56	7500	–	0,27	0,05	8000 k tonnes (2003)		2,45	5,38	2,46	122,06/122,75
Novolac glycidyl ether 3-Ring	NOGE	158,163-01-0	474,00	–	–	–	–	–		5,71 2,94	9,51 5,54	5,73 2,94	112,52 139,99
2,2-di(4methacryloxyphenyl) propane	BIS-DMA	3253-39-2	364,43	6200	–	–	–	–		5,76	9,18	5,76	108,77

[1] Optimized for lowest energy, with HyperChem 8.0 software, blue – carbon atoms, red – oxygen atoms, black – hydrogen atoms, yellow – chlorine atoms.
[2] Distance between central carbon atom and left branch oxygen atom forming phenol.
[3] Distance between both branches oxygen atoms forming phenols.
[4] Distance between central carbon atom and right branch oxygen atom forming phenol.
[5] Angle formed by spatial location of given three atoms (central carbon atom and two oxygen atoms forming phenol units).

Table 2
Selected information on content of BADGE-related compounds in various types of samples.

	Matrix type	Unit	Concentration of analytes determined										Final determination technique	Ref.
			BADGE	BADGE ·H_2O	BADGE ·$2H_2O$	BADGE ·2HCl	BADGE·HCl·H_2O	BFDGE	BFDGE ·$2H_2O$	BFDGE ·2HCl	DEP	DBP	DEHP		
Environmental samples	Indoor air	[μg/g]	0.36–0.98	1.79–3.85	0.04–0.85	7.15–7.3	3.57–4.01	3.57–4.01		1.79–2.43				LC-MS/MS	Xue et al. (2016)
	Indoor air	[μg/g]									1.549–1.580	318.3–346.1	92.299–84.52	GC–MS	Zhang et al. (2014)
	Indoor dust	[ng/g]	n.d. - 34.5	1.79–80.5	10.9–1630		2.49–417				9.49–362	73.7–4910	2080–76,500	GC–MS	Tran et al. (2016)
	Wastewater	[μg/L]	0.57 ± 0.04–96 ± 7					0.32 ± 0.05–92 ± 4						LC-FD	Ballesteros-Gómez et al. (2007)
	River water	[μg/L]	90 ± 4–95 ± 1					88 ± 2–91 ± 1						LC-FD	Ballesteros-Gómez et al. (2007)
	River water	[μg/L]	0.35					0.14						GC–MS/MS	Jiao et al. (2012)
	Biosolids	[ng/g]	n.d. - 1980	n.d. - 2090	n.d. - 2290	n.d. - 37.5	n.d. - 121	n.d. - 146	n.d. - 175	n.d. - 4510				LC-MS/MS	Xue et al. (2015)
	Sludge	[ng/mL]									126.18–893.17	537.4–1935.1	1853.64–9408.49	GC–MS	Gao et al. (2014)
Biological fluids	Urine	[ng/mL]	0.07–295		0.07–1450									LC-MS/MS	Cutanda et al. (2015)
	Urine	[ng/mL]	0.105–2.321	0.121–1.361	0.150–4.604		n.d.-3.412							LC-MS/MS	Wang et al. (2015)
	Plasma	[ng/mL]	n.d.	n.d. - 9.54	n.d. - 65.1	n.d.	n.d. - 1.41	23.3–180	n.d. - 6.65	n.d.				LC-MS/MS	Wang et al. (2015)
	Adipose	[ng/g]	n.d. - 5.16	n.d. - 4.33	n.d. - 45.4	n.d. - 3.11	n.d. - 28.2	19.1–4500	n.d. - 12.6	n.d. - 5.2				LC-MS/MS	Wang et al. (2015)

5 mL of growth medium was transferred to a labeled culture bottles with caps with a gas permeable filter, afterwards the yeast disks were sterilely transferred and placed on an orbital shaker set at 32 °C temperature and 100 rpm for 48 h. 100 μL of DMSO was added to each control vial containing standards: E2 (17β-estradiol control of YES agonist), DHT (5α-dihydrotestosterone control of YAS agonist), HT (4-hydroxytamoxifen control of YES antagonist), FL (flutamide control of YAS antagonist). Test plates were prepared in such a way that the controls were in duplicate in eight serial dilutions respectively:

YES Agonist plate E2 (min. concentration $1 \cdot 10^{-11}$ M, max. concentration $1 \cdot 10^{-8}$ M),

YES Antagonist plate HT (min. concentration $1 \cdot 10^{-8}$ M, max. concentration $1 \cdot 10^{-5}$ M, additionally in the entire plate E2 was present at constant concentration of $1 \cdot 10^{-9}$ M),

YAS Agonist plate DHT (min. concentration $1 \cdot 10^{-9}$ M, max. concentration $1 \cdot 10^{-6}$ M),

YAS Antagonist plate FL (min. concentration $1 \cdot 10^{-7}$ M, max. concentration $1 \cdot 10^{-4}$ M, additionally in the entire plate DHT was present at constant concentration of $3 \cdot 10^{-8}$ M).

Addition of E2 or DHT present at the same concentration to the entire YES or YAS antagonist plate, respectively, is intended to examine (confirm/deny) andro- and estrogenic antagonistic activity of samples. A substance with the antagonist properties competes with E2 or DHT present on the plate and binds to the receptor without inducing the expression of β-galactosidase. Without the enzyme substrate staining does not occur, however, if the test sample does not contain antagonistic substances, then E2 and DHT the present in the wells bind with the receptor expressing β-galactosidase and the staining of the substrate occurs.

80 μL of 6 mM CRPG dye was added to each assay well. The concentrations of stock (highest) solutions studied were greatly correlated to their solubility levels, the highest concentration of all analytes stock solution was prepared as 40 μg/mL and they were diluted in 1:10 ratio down to 0,039 μg/mL. This part of research – as can be easily noticed – did not aim to study mixtures behavior in case of Xenoscreen, it was performed to study plausible impact of different analytes from plasticizers group (at varying concentration levels) and relate it to environmentally levels as some of studies are summarized in Table 2. Serial dilutions of analytes were studied to detect a broad range of possible interactions. All of the studies on mixtures were performed in triplicates, furthermore controls were made for pure substances in duplicates. 100 μL of YES and YAS suspension of yeast culture (yeast cells density > 0.3 OD_{690}) was added into agonist and antagonist YES and YAS plates, respectively. Assay plates were sealed with semi-permeable membranes and placed in the bag zipper moistened with watered gauze on an orbital shaker for 48 h at 32 °C 100 rpm. After 48 h of incubation, a cell density (by OD) was read at a wavelength of 690 nm and color intensity at a wavelength of 570 nm was determined. Afterwards the activity of β-galactosidase was calculated as ratio of $[(OD_{570}\text{-}OD_{690})/OD_{690}]$.

2.4. Calculations of model deviation ratios (MDRs)

The two most exploited models for environmental hazard and risk assessments of mixtures are Concentration Addition (CA) and Independent Action (IA) (Wieczerzak et al., 2016b). These two approaches could assess combined toxicological effect of chemicals assuming similar mode of action (CA) or dissimilar mode of action (IA). In the environmental risk assessment CA models are more frequently applied since they are slightly more conservative than IA models and could be used as a precautious first tier for environmental hazard and risk assessment of mixtures, irrespective of the modes of action of their components.

Table 3
Summary information on selected studies using single cell lines and organisms from different trophic levels to determine toxicity of BADGE derivatives.

Analytes	Concentration range	Detection technique	Observed effects	Ref.
Model studies				
BADGE, BADGE·H_2O, BADGE·$2H_2O$, BADGE·2HCl	10–500 μg/plate	*E. coli* tryptophan reverse mutation assay	- BADGE was able induce mutagenic effects - The mutagenic effect of BADGE·H_2O was weaker than that obtained with BADGE - No mutagenic activity was found for BADGE·$2H_2O$ and BADGE·2HCl.	(Sueiro et al., 2006)
BFDGE	12.5–62.5 μg/ml	*Salmonella typhimurium* His(-) and *Escherichia coli* Trp- tests sister chromatid exchange and micronucleus tests in human lymphocytes	- BFDGE is able to induce mutagenic effects in bacterial strains - Induces an increase in the frequency of sister chromatid exchanges and micronuclei in human peripheral blood lymphocytes.	(Sueiro et al., 2003)
BADGE, BADGE·$2H_2O$	3 pM-300 μM	XenoScreen XL YES/YAS Assay (re-engineered *Saccharomyces cerevisiae*)	- Positive anti-estrogenic and anti-androgenic effect was noted for BADGE -BADGE·$2H_2O$ demonstrated no anti-estrogenic or anti-androgenic activities	(Fic et al., 2014)
BADGE, BADGE·$2H_2O$, BADGE·2HCl	10^{-14}–10^{-4} M	cell proliferation assay (T47D human breast cancer cells)	- Positive estrogenic activity was noted for BADGE·2HCl and BADGE·$2H_2O$	Nakazawa et al., 2002)
Samples of food contact materials				
Carton and polypropylene packaging (PET, PE, PP, PS)	–	YES/YAS (re-engineered *Saccharomyces cerevisiae*) CALUX	- Estrogenic activity was detected in some samples (highest effect observed for paper/aluminum/PE composite film for fatty products sample YES EEQ[a] = 59.6 ± 29.3 ng/L) - In the yeast oestrogen screen antagonistic effects was noted for foil and carton for milk product extract - None of the samples showed androgen activity in either YAS or AR CALUX	(Mertl et al., 2014)
Paper and paperboard packaging	–	Tests with human larynx carcinoma cell line (HEp-2) and metabolically competent mouse hepatoma cell line	- Samples showed marginal toxicity - Some toxic responses, mainly in the RNA- synthesis inhibition assay measuring sublethal effects were observed, - Cytotoxicity in HEp-2 cells was observed for water extracts	(Ozaki et al., 2004)
Plastic packaging	–	YES (re-engineered *Saccharomyces cerevisiae*)	- The highest percentage of oestrogen-positive samples was found in composite film extracts - The highest oestrogen activity determined for Paper/aluminum/PE composite film for fatty products YES (EEQ[a] = 59.6 ± 29.3 ng/L) - None of the PP samples showed estrogenic activity	(Galotto and Ulloa, 2010)

[a] EEQ - 17β-estradiol equivalent concentration.

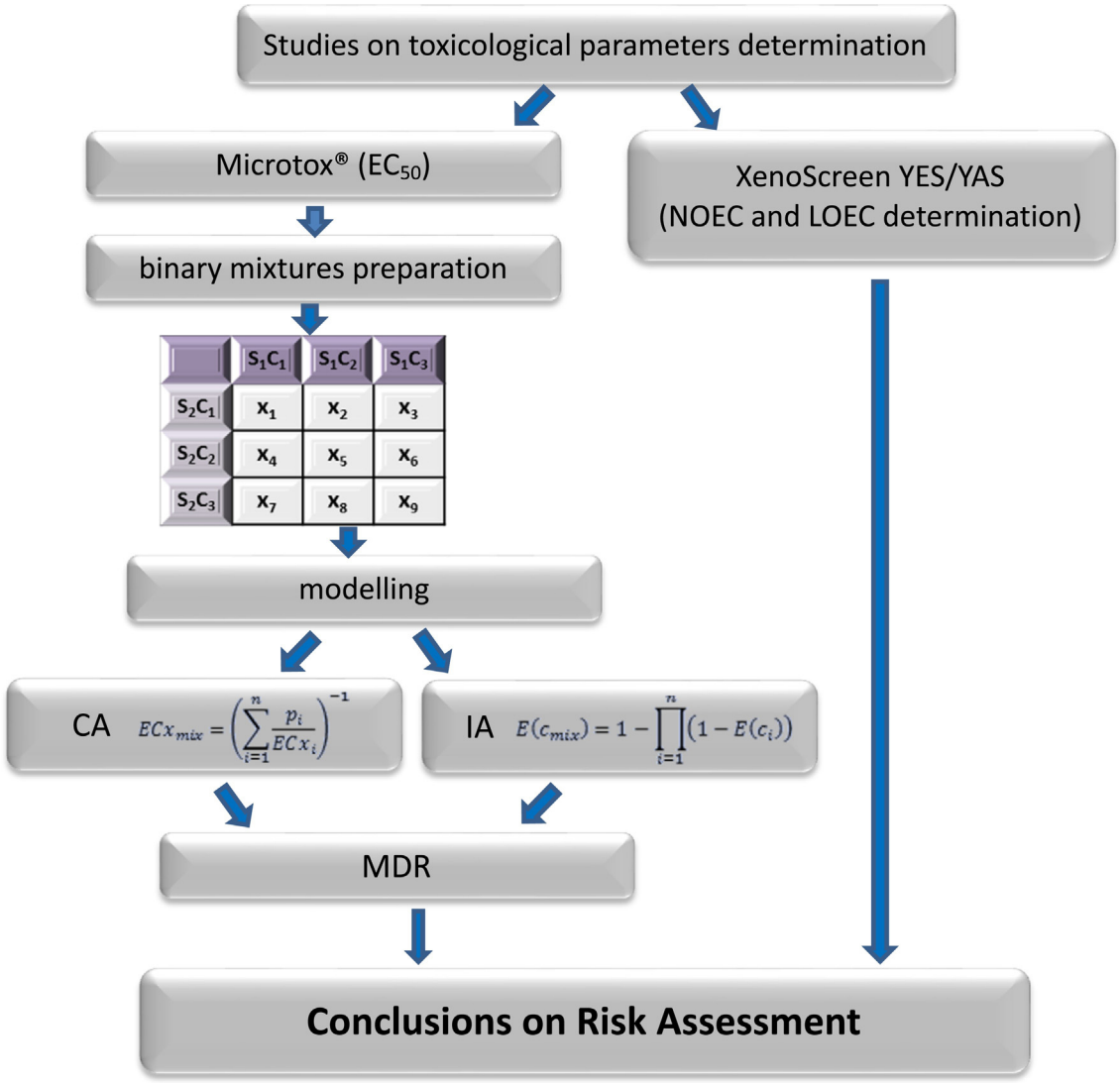


Fig. 1. Schematic presentation of research performed in the study.

Table 4
Concentration levels of bisphenol analogues studied during the research and EC_{50} values calculated for respective compounds.

Analyte	For Microtox®[a]					For XenoScreen YES/YAS®				
	Concentration ranges tested [μg/mL]	EC_{50}	c_1	c_2	c_3	Concentration ranges tested [μg/mL]	Effect			
							YES +	YES−	YAS +	YAS−
BADGE	6.49–65.45	>65.45[b]	–	–	–	0.039–40.00	>40.00^N	>40.00^N	>40.00^N	>40.00^N
BADGE·H_2O	6.49–65.45	39.25	12,95	25,91	39.25	0.039–40.00	>40.00^N	40.00^L	>40.00^N	>40.00^N
BADGE·$2H_2O$	8.12–81.82	59.09	19,50	39,00	59.09	0.039–40.00	>40.00^N	>40.00^N	>40.00^N	>40.00^N
BADGE·HCl	3.25–32.73	7.93	2,62	5,23	7.93	0.039–40.00	>40.00^N	4.00^L	>40.00^N	40.00^L
BADGE·2HCl	1.22–12.27	2.69	0,89	1,78	2.69	0.039–40.00	>40.00^N	4.00^L	>40.00^N	4.00^L
BADGE·H_2O·HCl	6.49–65.45	32.08	10,59	21,17	32.08	0.039–40.00	>40.00^N	40.00^L	>40.00^N	40.00^L
BFDGE	8.12–81.82	64.38	21,25	42,49	64.38	0.039–40.00	>40.00^N	>40.00^N	>40.00^N	40.00^L
BFDGE·$2H_2O$	12.99–130.91	96.94	31,99	63,98	96.94	0.039–40.00	>40.00^N	4.00^L	>40.00^N	40.00^L
BFDGE·2HCl	4.87–49.09	6.31	2,08	4,16	6.31	0.039–40.00	>40.00^N	>40.00^N	>40.00^N	>40.00^N
DEP	12.99–130.91	117.49	38,77	77,54	117.49	0.039–40.00	>40.00^N	>40.00^N	>40.00^N	40.00^L
DBP	6.49–65.45	56.97	18,80	37,60	56.97	0.039–40.00	>40.00^N	>40.00^N	>40.00^N	>40.00^N
DEHP	6.49–65.45	>65.45[#]	–	–	–	0.039–40.00	>40.00^N	>40.00^N	>40.00^N	>40.00^N
NOGE	6.49–65.45	>65.45[#]	–	–	–	0.039–40.00	>40.00^N	>40.00^N	>40.00^N	>40.00^N
BIS-DMA	6.49–65.45	>65.45[#]	–	–	–	0.039–40.00	4.00^L	>40.00^N	>40.00^N	>40.00^N

[a] c_1, c_2 and c_3 stand for 33, 66 and 100% of EC_{50} of respective analyte.
[b] Solubility limit reached under conditions of the experiment, L – LOEC, N – NOEC.

In this study the combined toxicological effect of mixture was assessed by CA model using Eq. (1) [43]:

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

where:

- ECx_{mix} is the total concentration of the mixture that causes x effect,
- p_i indicates the proportion of component i in the mixture,
- n indicates the number of components in the mixture,
- ECx_i indicates the concentration of component i that would cause x effect.

The independent action (IA)model is used to test toxicants in a mixture for a dissimilar mode of action. The concept is that they act independently. In fact the IA model is a statistical approach to predict the chance that one of multiple events will occur. The total mixture effect is calculated using Eq. (2):

$$E(c_{mix}) = 1 - \prod_{i=1}^{n}(1 - E(c_i)) \quad (2)$$

where:

- $E(c_{mix})$ is the total concentration of the mixture,
- $E(c_i)$ is the concentration expected from component i.

The CA model does not count for possible interaction between different chemicals in the mixture and deviations of tested mixture toxicity from predicted one could be evidence for synergistic or antagonistic interaction between chemicals. To outline significant deviations (interactions between chemicals) the model deviation ratio (MDR) approach proposed by Belden et al. (2007) is applied. MDR (unitless) is defined as (Eq. (3)):

$$MDR = \frac{Expected\ toxicity}{Observed\ toxicity} \quad (3)$$

where:

- *Expected toxicity* is the effective toxicity (inhibition of endpoint observed) for the mixture predicted by CA/IA model,
- *Observed toxicity* is the effective toxicity (inhibition of endpoint observed) for the mixture obtained from toxicity testing.

The MDR values are easily applicable to reflect impact of toxicants mixture when compared to predictive models. MDRs can be also presented in a plot form on logarithmic scale to visualize the predicted toxicity in comparison to observed one. The mixtures with MDR values falling outside the range from 0.5 to 2.0 have high probability for biologically significant synergistic or antagonistic interactions between chemicals. The underestimated or overestimated toxicity mixtures close to these levels also most likely include possible synergistic or antagonistic interactions (Belden et al., 2007; Wieczerzak et al., 2016b). In current research it was arbitrarily assumed that MDR falling within 0.50–0.71 and 1.40–2.00 justify the concluding on, respectively, possible under- and overestimation of presented models.

2.5. Quality assurance/quality control

For quality assurance of running the proper test, the following parameters according to the manufacturers' guidelines were used: for Microtox®, I_0 of bacterial suspension >70 U (chromium sulfate was used as a positive control in the bacterial stock suspension test run); for Xenoscreen YES/YAS, the OD_{690} of yeast cultures should be >0.3. In all cases presented, these factors were fulfilled.

3. Results and discussion

Table 4 shows the results of the studies on toxicity and endocrine potential as well the subsequent selection of C_1, C_2 and C_3 (being 33, 66 and 100% of EC_{50} of the respective analyte) and the determination of the impact of their co-presence in binary mixture on toxicity levels. Data on LOEC (lowest observable effect concentration) and NOEC (no-observed effect concentration) of given chemicals with respect to Xenoscreen YES/YAS are also shown. As can be seen in prevailing number of cases no-effect could be reached neither for androgenic nor estrogenic activity of chemicals studied under concentration levels studied. In this study it was found that agonistic estrogenic impact was provoked only by BIS-DMA (for concentrations of stock solutions at levels ≥4.00 μg/mL) while none of chemicals studied showed agonistic androgenic action. Androgenic antagonistic activity was exhibited by all hydrochloric derivatives of BADGE as well as BFDGE and BFDGE·$2H_2O$. BADGE·2HCl was observed to be the most potent YAS- substance studied showing its impact already at ≥4.00 μg/mL. Estrogenic antagonistic action was seen in cases of BFDGE·$2H_2O$, BADGE·H_2O and all its hydrochloric derivatives even at ≥4.00 μg/mL what confirms importance of risk posed by given analytes. The problem with performing studies on solutions at higher concentration levels would be connected with reaching and/or overcoming solubility limits of given analytes in water solutions. Certainly the problem may be reduced by adding other solvents (e.g. ethanol, methanol, DMSO etc.) to reaction mixture to increase solubility of analytes although such action does not reflect real situation that may occur under environmental conditions and may impact well-being of yeasts used for research thus impacting the final results in unpredictable and uncontrolled way.

3.1. CA studies

Analyses of MDR variations in CA modelling reveal some interesting dependencies and trends (Table 5). BADGE·H_2O has exhibited a synergistic impact in all cases of binary mixtures, especially at the lowest concentration levels, which is of great importance in the case of environmental threat assessment. With the growing content of BADGE·H_2O in a mixture, the trend of synergism weakens, but is still underestimated in most cases. Interestingly, in mixtures with DEP, the CA model proves that the mode of action of an analyte is independent of its nature. In the case of DBP, which is structurally similar to DEP, one can observe synergistic phenomena.

In most cases, the hydrochloric derivative of BADGE, with other substances in binary mixtures, shows independent behavior, with MDR values oscillating approximately 1.00. Only in the case of the BADGE·H_2O·HCl mixture are synergistic and underestimated behaviors observed.

BADGE·$2H_2O$ exhibits similar behavior to BADGE·H_2O. An overwhelming number of binary mixture cases shows synergistic or underestimated action, with the only exception being the mixture with DEP, where independent action is confirmed with an increasing content of mixture ingredients. The same behavior is observed for almost all mixtures of DEP; the only exception is observed when this analyte is mixed with BADGE·H_2O·HCl.

BFDGE is an interesting example of a compound posing a great threat to the environment (just like BADGE·H_2O) as it shows a synergistic response in almost all of the mixtures studied. Only in the case of BFDGE/BADGE·H_2O·HCl is the independent mode of action of ingredients observed, with MDR values being slightly lower than 1.00.

BFDGE·H_2O shows an independent mode of action with its hydrochloric derivatives, DEP and BADGE·H_2O·HCl. Interestingly, the mixture with DBP shows many cases that are synergistic and underestimated.

Table 5
MDR values for bisphenol A derivatives binary mixtures toxicity studies (for Concentration Addition modelling) (red – synergism, blue – antagonism, green – overestimation, yellow – underestimation, C1, C2 and C3 stand for 33, 66 and 100% of EC_{50} of respective analogue as presented in Table 4.

		BADGE·H_2O																														
		C_1	C_2	C_3																												
C_1	BADGE ·HCl	0,29	0,68	0,94																												
C_2		0,57	0,38	1,54	BADGE·HCl																											
C_3		0,58	0,49	0,46	C_1	C_2	C_3																									
C_1	BADGE·$2H_2O$	0,15	0,50	0,39	0,56	0,71	0,63																									
C_2		0,58	0,41	0,89	0,80	0,82	0,75	BADGE·$2H_2O$																							
C_3		0,73	0,68	0,30	0,89	1,06	0,61	C_1	C_2	C_3																					
C_1	BFDGE	0,27	0,32	0,42	1,11	1,18	1,19	0,27	0,42	0,54																					
C_2		0,36	0,39	0,46	1,51	1,32	1,54	0,33	0,45	0,53	BFDGE																				
C_3		0,45	0,48	0,51	1,34	1,24	0,91	0,42	0,53	0,57	C_1	C_2	C_3																		
C_1	BFDGE·$2H_2O$	0,36	0,59	0,64	0,60	0,62	0,67	0,35	0,62	0,66	0,37	0,46	0,64																		
C_2		0,66	0,55	0,72	0,78	0,79	0,93	0,61	0,53	0,76	0,42	0,50	0,64	BFDGE·$2H_2O$																	
C_3		0,71	0,64	0,55	0,84	0,95	0,69	0,74	1,19	0,44	0,54	0,56	0,63	C_1	C_2	C_3															
C_1	BFDGE·2HCl	0,23	0,47	0,62	0,61	0,84	0,89	0,25	0,80	0,79	0,26	0,32	0,41	0,63	0,93	0,91															
C_2		0,32	0,36	0,85	0,77	0,81	0,84	0,32	0,63	0,67	0,36	0,40	0,47	0,69	0,72	1,14	BFDGE ·2HCl														
C_3		0,35	0,40	0,50	0,81	1,12	0,70	0,34	1,29	0,49	0,48	0,49	0,55	0,68	0,73	0,7 5	C_1	C_2	C_3												
C_1	BADGE·2HCl	0,20	0,37	0,53	0,66	0,97	0,95	0,34	0,76	1,39	0,24	0,33	0,52	0,68	0,99	1,07	0,31	0,64	0,67												
C_2		0,25	0,34	0,71	0,74	1,02	0,95	0,80	0,52	1,00	0,24	0,33	0,53	0,83	0,88	1,23	0,43	0,49	0,95	BADGE·2HCl										
C_3		0,23	0,32	0,50	0,75	1,00	0,61	0,56	1,23	0,50	0,25	0,34	0,51	0,82	0,85	0,74	0,42	0,53	0,61	C_1	C_2	C_3								
C_1	BADGE ·H_2O·HCl	0,26	0,44	0,56	0,40	0,52	0,61	0,40	0,64	0,82	0,76	0,85	0,89	0,55	0,67	0,75	0,33	0,52	0,51	0,27	0,49	0,39									
C_2		0,44	0,43	0,91	0,80	0,65	0,66	0,74	0,56	0,65	0,76	0,81	0,86	0,82	0,73	0,98	0,90	0,70	1,54	0,85	0,72	1,20	BADGE·H_2O·HCl							
C_3		0,45	0,47	0,58	0,79	1,27	0,73	0,66	0,94	0,51	0,90	0,93	0,91	0,69	0,65	0,66	0,71	0,64	0,63	0,71	0,62	0,55	C_1	C_2	C_3						
C_1	DEP	0,50	0,93	0,83	0,66	0,78	0,69	0,62	0,83	0,87	0,52	0,57	0,64	0,58	0,82	0,84	0,68	0,75	0,67	0,58	0,85	0,72	0,38	0,52	0,49						
C_2		0,91	0,62	1,46	0,72	0,74	0,83	0,94	0,77	1,11	0,56	0,59	0,63	0,77	0,74	1,01	1,00	0,70	1,01	0,90	0,74	1,19	0,54	0,50	0,64	DEP					
C_3		1,12	0,92	0,58	0,86	0,81	0,66	0,95	0,84	0,66	0,64	0,67	0,66	0,85	0,80	0,68	0,94	0,92	0,64	1,13	0,94	0,65	0,75	0,68	0,61	C_1	C_2	C_3			
C_1	DBP	0,27	0,42	0,45	1,22	1,13	1,06	0,51	0,71	0,73	0,43	0,53	0,70	0,49	0,72	0,73	0,56	0,61	0,69	0,58	0,86	0,61	0,42	0,69	0,71	0,62	0,95	1,06			
C_2		0,38	0,39	0,62	0,89	0,99	1,04	0,66	0,66	0,85	0,43	0,49	0,63	0,62	0,62	0,81	0,70	0,59	0,96	0,71	0,64	0,75	0,58	0,51	0,95	0,68	1,13	1,27			
C_3		0,58	0,53	0,54	1,01	1,03	0,56	0,93	0,92	0,63	0,58	0,61	0,63	0,79	0,73	0,60	0,81	0,86	0,55	0,95	0,94	0,58	1,04	0,82	0,58	0,80	1,26	0,65			

Table 6
MDR values for bisphenol A derivatives binary mixtures toxicity studies (for Independent Action modelling) (red – synergism, blue – antagonism, green – overestimation, yellow – underestimation, C1, C2 and C3 stand for 33, 66 and 100% of EC_{50} of respective analogue as presented in Table 4.

		BADGE·H_2O		
		C_1	C_2	C_3
C_1	BADGE ·HCl	0,53	1,05	1,24
C_2		1,15	0,65	2,18
C_3		1,24	0,90	0,68
C_1	BADGE ·$2H_2O$	0,42	1,66	1,43
C_2		1,01	0,77	1,79
C_3		1,18	1,21	0,57
C_1	BFDGE	0,46	0,55	0,70
C_2		0,56	0,62	0,74
C_3		0,66	0,73	0,76
C_1	BFDGE ·$2H_2O$	0,62	0,99	1,00
C_2		1,03	0,87	1,08
C_3		1,03	0,95	0,79
C_1	BFDGE ·2HCl	0,54	0,85	0,86
C_2		0,79	0,70	1,24
C_3		0,97	0,85	0,79
C_1	BADGE ·2HCl	0,53	0,72	0,76
C_2		0,80	0,76	1,13
C_3		0,84	0,76	0,89
C_1	BADGE ·H_2O·HCl	0,55	0,81	0,82
C_2		0,90	0,81	1,38
C_3		0,92	0,88	0,88
C_1	DEP	0,76	1,47	1,32
C_2		1,26	0,91	2,23
C_3		1,44	1,24	0,80
C_1	DBP	0,47	0,72	0,71
C_2		0,60	0,63	0,95
C_3		0,87	0,80	0,78

		BADGE ·HCl		
		C_1	C_2	C_3
C_1	BADGE ·$2H_2O$	0,84	1,10	0,99
C_2		1,06	1,11	1,04
C_3		1,11	1,36	0,80
C_1	BFDGE	1,26	1,40	1,39
C_2		1,63	1,46	1,70
C_3		1,42	1,34	0,98
C_1	BFDGE ·$2H_2O$	0,94	1,04	1,12
C_2		1,12	1,23	1,44
C_3		1,15	1,39	0,83
C_1	BFDGE ·2HCl	0,90	1,14	1,17
C_2		1,09	1,10	1,12
C_3		1,09	1,46	0,91
C_1	BADGE ·2HCl	0,97	1,30	1,22
C_2		1,11	1,41	1,26
C_3		1,13	1,40	0,83
C_1	BADGE ·H_2O·HCl	0,65	0,87	1,00
C_2		1,06	0,88	0,90
C_3		0,95	1,56	0,91
C_1	DEP	1,09	1,36	1,19
C_2		1,15	1,26	1,41
C_3		1,21	1,20	0,97
C_1	DBP	1,65	1,58	1,50
C_2		1,26	1,44	1,55
C_3		1,39	1,45	0,80

		BADGE·$2H_2O$		
		C_1	C_2	C_3
C_1	BFDGE	0,48	0,73	0,91
C_2		0,53	0,76	0,91
C_3		0,64	0,81	0,88
C_1	BFDGE ·$2H_2O$	0,68	1,08	1,10
C_2		1,12	0,88	1,26
C_3		1,32	1,93	0,80
C_1	BFDGE ·2HCl	0,56	1,26	1,03
C_2		0,80	1,08	0,93
C_3		1,21	2,85	0,79
C_1	BADGE ·2HCl	0,70	1,28	1,85
C_2		2,35	1,18	1,61
C_3		1,84	3,13	0,86
C_1	BADGE ·H_2O·HCl	0,75	1,11	1,27
C_2		1,31	0,97	1,02
C_3		1,12	1,62	0,80
C_1	DEP	0,95	1,31	1,34
C_2		1,35	1,16	1,71
C_3		1,26	1,16	0,95
C_1	DBP	0,86	1,15	1,13
C_2		1,04	1,03	1,31
C_3		1,37	1,39	0,89

		BFDGE		
		C_1	C_2	C_3
C_1	BFDGE ·$2H_2O$	0,51	0,72	0,92
C_2		0,66	0,77	0,97
C_3		0,80	0,83	0,89
C_1	BFDGE ·2HCl	0,47	0,54	0,67
C_2		0,59	0,64	0,74
C_3		0,72	0,75	0,80
C_1	BADGE ·2HCl	0,54	0,60	0,71
C_2		0,62	0,67	0,77
C_3		0,74	0,77	0,82
C_1	BADGE ·H_2O·HCl	0,99	1,00	1,01
C_2		1,01	0,99	1,01
C_3		1,01	1,02	0,99
C_1	DEP	0,88	0,92	0,97
C_2		0,90	0,93	0,97
C_3		0,93	0,96	0,95
C_1	DBP	0,69	0,80	0,97
C_2		0,69	0,77	0,95
C_3		0,85	0,91	0,89

		BFDGE·$2H_2O$		
		C_1	C_2	C_3
C_1	BFDGE ·2HCl	0,88	1,19	1,07
C_2		1,03	0,98	1,41
C_3		1,08	1,05	0,96
C_1	BADGE ·2HCl	0,92	1,23	1,26
C_2		1,24	1,18	1,54
C_3		1,24	1,16	0,93
C_1	BADGE ·H_2O·HCl	0,85	0,99	0,99
C_2		1,27	1,13	1,35
C_3		1,04	1,03	0,93
C_1	DEP	0,90	1,24	1,23
C_2		1,13	1,10	1,49
C_3		1,16	1,12	0,96
C_1	DBP	0,82	1,11	1,06
C_2		0,98	0,94	1,18
C_3		1,18	1,07	0,85

		BFDGE ·2HCl		
		C_1	C_2	C_3
C_1	BADGE ·2HCl	0,64	1,00	0,90
C_2		0,87	0,79	1,32
C_3		0,88	0,88	0,86
C_1	BADGE ·H_2O·HCl	0,54	0,90	1,03
C_2		1,24	1,03	2,43
C_3		1,11	1,34	1,25
C_1	DEP	1,11	1,34	1,25
C_2		1,47	1,11	1,67
C_3		1,24	1,29	0,93
C_1	DBP	1,04	1,22	1,44
C_2		1,14	1,01	1,76
C_3		1,18	1,33	0,90

		BADGE ·2HCl		
		C_1	C_2	C_3
C_1	BADGE ·H_2O·HCl	0,45	1,08	0,99
C_2		1,15	1,20	2,26
C_3		0,87	0,85	0,82
C_1	DEP	0,95	1,48	1,37
C_2		1,29	1,13	1,96
C_3		1,48	1,28	0,94
C_1	DBP	0,96	1,63	1,29
C_2		1,03	1,04	1,33
C_3		1,23	1,32	0,89

		BADGE·H_2O·HCl		
		C_1	C_2	C_3
C_1	DEP	0,66	0,98	0,92
C_2		0,80	0,81	1,07
C_3		1,01	0,97	0,89
C_1	DBP	0,68	1,30	1,17
C_2		0,89	0,83	1,56
C_3		1,43	1,16	0,87

		DEP		
		C_1	C_2	C_3
C_1	DBP	1,09	1,71	1,37
C_2		1,17	1,90	1,72
C_3		1,37	1,73	0,87

HCl analogues of BADGE and BFDGE exhibit a synergistic impact on bacterial metabolic processes, as in the case of mixtures with DBP. The same holds true for the hydrochloric derivatives of BADGE and DBP. The mixtures of DEP with BADGE·H_2O·HCl are the only ones among the DEP-2nd ingredients that show predominantly synergistic action and underestimation of its toxic potential.

3.2. IA studies

BADGE·H_2O shows independent behavior in most cases. The model predominantly and properly describes a real situation; however, some discrepancies are observed in mixtures with BADGE·H2O (Table 6). In mixtures with DEP, overestimation and antagonism are observed. The hydrochloric derivatives of BADGE are correctly modeled with IA (MDR values ~1.00) or slightly overestimated. Interestingly, BADGE·H_2O is the only case of a mixture with BFDGE showing synergism, suggesting that it is a great environmental threat when present in different environmental compartments. BFDGE, also in mixture with its chlorinated form, shows synergism and underestimation. With BADGE·H_2O·HCl, DEP and DBP the model shows a mode of action independent of these compound mixtures.

BFDGE·$2H_2O$ in binary mixtures with BFDGE·2HCl, BADGE·2HCl, BADGE·H_2O·HCl, DEP and DBP is correctly modeled, as MDR values are close to 1.00. A similar situation holds true for the BFDGE·2HCl. In the case of BADGE·2HCl, synergism is observed at the lowest concentration levels and is proof of its threat when present at low concentration levels in different environmental compartments. Similar to the case of CA DEP, an independent mode of action has the tendency to be antagonistic in almost all cases with DBP.

3.3. MDR uncertainties

MDR values presented enable providing uncertainties for frequency distribution of results given (please refer to Table 7. for details) and determining safety factors in case of pollutants presence stated in complex mixtures. The CA model has again the tendency for overprediction – it confirms to be systematic behavior of model but from environmental point of view it can be considered as an advantage as one can easily target the most toxic/dangerous components of mixture in this way.

4. Conclusions

Complex studies on the impacts of pollutants and/or environmentally benign substances are currently and quickly developing within numerous scientific centres. Still, the significance and proper assessment of such mixtures will probably be a challenge to all environmental professionals dealing with health risk assessment. In the study presented, a quick and reliable method for modelling BADGE and BFDGE derivatives toxicity and endocrine potential is given. To a great extent, the mixtures show an independent or synergistic mode of action, especially in the case of the hydrochloric derivatives of the analytes of interest. In the studies presented, the MDR approach enabled quantitative description/scaling of independent, synergistic or antagonistic effects taking place when the analytes studied are present in binary mixtures. Summarizing both the literature studies and results of research conducted we could say that:

There is a high probability of BADGE and BFDGE together with their metabolites/transformation products will be routine constituents of some wastewater effluent (Ballesteros-Gómez et al., 2007; Jiao et al., 2012),

Some previous literature reports up to 91 μg/L of BADGE in wastewater samples (Ballesteros-Gómez et al., 2007),
We were able to provide evidence for some estrogenic potential of the BADGE and BFDGE and their metabolites/transformation products,
More field measurements are needed to assess the holistic risk of analytes of interest.

The data shown constitute a record that may deliver decisive bodies new tools for implementing legal regulations in order to better deal with this type of pollution. The concentration ranges studied in several cases (BADGE, BFDGE and their HCl derivatives) reach similar levels as environmentally relevant ones. What is more risky, from an environmental point of view is that these analytes exhibit a tendency to act in a synergic manner and may be present in some yet unexamined utensils or compartments (e.g. food, food contact materials, toys, medical devices, wastewater effluents, accidental spills etc.) (Míguez et al., 2012; Xue et al., 2017, 2015) at much higher concentration levels. Unintended events, such as an accidental spill of BADGE and BFDGE, may pose an additional threat to ecosystems. In all of these cases, the results presented here may give simple clues and information on the possibility of predicting the combined effects of given stressors to endocrine and bacterial responses. Future research will be directed toward studying the synergistic/antagonistic impact of a wider spectrum of analytes belonging to the bisphenol group (including those not yet used industrially) and higher-order mixtures (e.g., 3 × 3, 4 × 4, etc.) to reflect more scenarios that may occur in the environment and poison or degrade it. Similar studies will be conducted for other bioassays, including chronic endpoints and different chemicals of environmental relevance and XenoScreen YES/YAS to learn on plausible synergistic/antagonistic activity of analytes of interest when present at lower concentration levels (then studied in current research) in complex environmentally relevant mixtures.

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References

Al-Natsheh, M., Alawi, M., Fayyad, M., Tarawneh, I., 2015. Simultaneous GC-MS determination of eight phthalates in total and migrated portions of plasticized polymeric toys and childcare articles. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 985, 103–109.

Bakir, A., Rowland, S.J., Thompson, R.C., 2012. Competitive sorption of persistent organic pollutants onto microplastics in the marine environment. Mar. Pollut. Bull. 64, 2782–2789.

Ballesteros-Gómez, A., Ruiz, F.J., Rubio, S., Pérez-Bendito, D., 2007. Determination of bisphenols A and F and their diglycidyl ethers in wastewater and river water by coacervative extraction and liquid chromatography-fluorimetry. Anal. Chim. Acta 603, 51–59.

Table 7
Percentile values for model deviation ratios (MDR) and the number of cases for each group of CA and IA experiments of bisphenol A diglycidyl ether analogues toxicity studies.

model	No. of cases				Percentile			
	Synergism	Under-estimation	Over-estimation	antagonism	80	90	95	99
CA	91	146	5	0	0.900	1.010	1.190	1.513
IA	6	38	43	7	1.304	1.460	1.688	2.360

Belden, J.B., Gilliom, R.J., Lydy, M.J., 2007. How well can we predict the toxicity of pesticide mixtures to aquatic life? Integr. Environ. Assess. Manag. 3, 364–372.
Chang, Y., Nguyen, C., Paranjpe, V.R., Gilliland, F., Zhang, J., 2014. Analysis of bisphenol A diglycidyl ether (BADGE) and its hydrolytic metabolites in biological specimens by high-performance liquid chromatography and tandem mass spectrometry. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 965, 33–38.
Cunha, S.C., Cunha, C., Ferreira, A.R., Fernandes, J.O., 2012. Determination of bisphenol A and bisphenol B in canned seafood combining QuEChERS extraction with dispersive liquid-liquid microextraction followed by gas chromatography-mass spectrometry. Anal. Bioanal. Chem. 404, 2453–2463.
Cutanda, F., Koch, H.M., Esteban, M., Sanchez, J., Angerer, J., Castano, A., 2015. Urinary levels of eight phthalate metabolites and bisphenol A in mother-child pairs from two Spanish locations. Int. J. Hyg. Environ. Health 218, 47–57.
European Parliament, 2011. Commission directive 2011/8/EU. Off. J. Eur. Communities 29–32.
Fiandanese, N., Borromeo, V., Berrini, A., Fischer, B., Schaedlich, K., Schmidt, J.S., Secchi, C., Pocar, P., 2016. Maternal exposure to a mixture of di(2-ethylhexyl) phthalate (DEHP) and polychlorinated biphenyls (PCBs) causes reproductive dysfunction in adult male mouse offspring. Reprod. Toxicol. 65, 123–132.
Fic, A., Žegura, B., Gramec, D., Mašič, L.P., 2014. Estrogenic and androgenic activities of TBBA and TBMEPH, metabolites of novel brominated flame retardants, and selected bisphenols, using the XenoScreen XL YES/YAS assay. Chemosphere 112, 362–369.
Gallart-Ayala, H., Moyano, E., Galceran, M.T., 2011. Fast liquid chromatography-tandem mass spectrometry for the analysis of bisphenol A-diglycidyl ether, bisphenol F-diglycidyl ether and their derivatives in canned food and beverages. J. Chromatogr. A 1218, 1603–1610.
Gallo, P., Di Marco Pisciottano, I., Esposito, F., Fasano, E., Scognamiglio, G., Mita, G.D., Cirillo, T., 2017. Determination of BPA, BPB, BPF, BADGE and BFDGE in canned energy drinks by molecularly imprinted polymer cleaning up and UPLC with fluorescence detection. Food Chem. 220, 406–412.
Galotto, M., Ulloa, P., 2010. Detection and identification of oestrogen-active substances in plastic food packaging migrates. Packag. Technol. Sci. 23, 253–266.
Gao, D., Li, Z., Wen, Z., Ren, N., 2014. Occurrence and fate of phthalate esters in full-scale domestic wastewater treatment plants and their impact on receiving waters along the Songhua River in China. Chemosphere 95, 24–32.
Geueke, B., Muncke, J., 2017. Substances of very high concern in food contact materials: migration and regulatory background. Packag. Technol. Sci. http://dx.doi.org/10.1002/pts.2288 (in press).
Grob, K., Camus, P., Gontard, N., Hoellinger, H., Joly, C., Macherey, A.C., Masset, D., Nesslany, F., Régnier, J.F., Riquet, A.M., Saillard, P., Ribera, D., 2010. Need for a better safety evaluation of food contact materials produced from resins. Food Control 21, 763–769.
Hu, Y., Wang, R., Xiang, Z., Qian, W., Han, X., Li, D., 2014. Antagonistic effects of a mixture of low-dose nonylphenol and Di-N-butyl phthalate (monobutyl phthalate) on the sertoli cells and serum reproductive hormones in prepubertal male rats in vitro and in vivo. PLoS One 9, 1–9.
Jiao, Y., Ding, L., Fu, S., Zhu, S., Li, H., Wang, L., 2012. Determination of bisphenol A, bisphenol F and their diglycidyl ethers in environmental water by solid phase extraction using magnetic multiwalled carbon nanotubes followed by GC–MS/MS. Anal. Methods 4, 291–298.
Lapviboonsuk, J., Leepipatpiboon, N., Harman-Fetcho, J.A., Donoghue, D.J., Roncari, A., Rudolph, T., Anklam, E., 2014. A simple method for the determination of bisphenol A diglycidyl ether and its derivatives in canned fish. Anal. Methods 6, 5666.
Li, X., Yin, P., Zhao, L., 2017. Effects of individual and combined toxicity of bisphenol a, dibutyl phthalate and cadmium on oxidative stress and genotoxicity in HepG 2 cells. Food Chem. Toxicol. 105, 73–81.
Mertl, J., Kirchnawy, C., Osorio, V., Grininger, A., Richter, A., Bergmair, J., Pyerin, M., Washüttl, M., Tacker, M., 2014. Characterization of estrogen and androgen activity of food contact materials by different in vitro bioassays (YES, YAS, ERα and AR CALUX) and chromatographic analysis (GC–MS, HPLC-MS). PLoS One 9.
Míguez, J., Herrero, C., Quintás, I., Rodríguez, C., Gigosos, P.G., Mariz, O.C., 2012. A LC-MS/MS method for the determination of BADGE-related and BFDGE-related compounds in canned fish food samples based on the formation of [M+NH(4)](+) aducts. Food Chem. 135, 1310–1315.
Møller, L., Leck, F., Og, F., Bo, P., Dhi, L., 2012. Survey of Bisphenol A and Bisphenol-A-Diglycidylether Polymer. 978-87-93026-14-8pp. 1–155.
Nakazawa, H., Yamaguchi, A., Inoue, K., Yamazaki, T., Kato, K., Yoshimura, Y., Makino, T., 2002. In vitro assay of hydrolysis and chlorohydroxy derivatives of bisphenol A diglycidyl ether for estrogenic activity. Food Chem. Toxicol. 40, 1827–1832.
Ozaki, A., Yamaguchi, Y., Fujita, T., Kuroda, K., Endo, G., 2004. Chemical analysis and genotoxicological safety assessment of paper and paperboard used for food packaging. Food Chem. Toxicol. 42, 1323–1337.
Pérez-Palacios, D., Fernández-Recio, M.Á., Moreta, C., Tena, M.T., 2012. Determination of bisphenol-type endocrine disrupting compounds in food-contact recycled-paper materials by focused ultrasonic solid-liquid extraction and ultra performance liquid chromatography-high resolution mass spectrometry. Talanta 99, 167–174.
Petersen, H., Biereichel, A., Burseg, K., Simat, T.J., Steinhart, H., 2008. Bisphenol A diglycidyl ether (BADGE) migrating from packaging material "disappears" in food: reaction with food components. Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 25, 911–920.
Ramirez, T., Buechse, A., Dammann, M., Melching-Kollmu, S., Woitkowiak, C., van Ravenzwaay, B., 2014. Effect of estrogenic binary mixtures in the yeast estrogen screen (YES). Regul. Toxicol. Pharmacol. 70, 286–296.
Rochman, C.M., Hoh, E., Hentschel, B.T., Kaye, S., 2013. Long-term field measurement of sorption of organic contaminants to five types of plastic pellets: implications for plastic marine debris. Environ. Sci. Technol. 47, 1646–1654.
Satoh, K., Ohyama, K., Aoki, N., Iida, M., Nagai, F., 2004. Study on anti-androgenic effects of bisphenol a diglycidyl ether (BADGE), bisphenol F diglycidyl ether (BFDGE) and their derivatives using cells stably transfected with human androgen receptor, AR-EcoScreen. Food Chem. Toxicol. 42, 983–993.
Suciu, N.A., Tiberto, F., Vasileiadis, S., Lamastra, L., Trevisan, M., 2013. Recycled paper-paperboard for food contact materials: contaminants suspected and migration into foods and food simulant. Food Chem. 141, 4146–4151.
Sueiro, R.A., Suárez, S., Araujo, M., Garrido, M.J., 2003. Mutagenic and genotoxic evaluation of bisphenol F diglycidyl ether (BFDGE) in prokaryotic and eukaryotic systems. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 536, 39–48.
Sueiro, R.A., Suárez, S., Araujo, M., Garrido, M.J., 2006. Study on mutagenic effects of bisphenol a diglycidyl ether (BADGE) and its derivatives in the Escherichia Coli tryptophan reverse mutation assay. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 609, 11–16.
Thompson, R.C., Moore, C.J., vom Saal, F.S., Swan, S.H., 2009. Plastics, the environment and human health: current consensus and future trends. Philos. Trans. R. Soc. Lond. B Biol. Sci. 364, 2153–2166.
Tran, T.M., Minh, T.B., Kumosani, T.A., Kannan, K., 2016. Occurrence of phthalate diesters (phthalates), p-hydroxybenzoic acid esters (parabens), bisphenol A diglycidyl ether (BADGE) and their derivatives in indoor dust from Vietnam: implications for exposure. Chemosphere 144, 1553–1559.
Wang, L., Xue, J., Kannan, K., 2015. Widespread occurrence and accumulation of bisphenol a diglycidyl ether (BADGE), bisphenol F diglycidyl ether (BFDGE) and their derivatives in human blood and adipose fat. Environ. Sci. Technol. 49, 3150–3157.
Wieczerzak, M., Kudłak, B., Yotova, G., Nedyalkova, M., Tsakovski, S., Simeonov, V., Namieśnik, J., 2016a. Modeling of pharmaceuticals mixtures toxicity with deviation ratio and best-fit functions models. Sci. Total Environ. 571, 259–268.
Wieczerzak, M., Namieśnik, J., Kudłak, B., 2016b. Bioassays as one of the green chemistry tools for assessing environmental quality: a review. Environ. Int. 94, 341–361.
Xue, J., Kannan, K., 2016. Novel finding of widespread occurrence and accumulation of bisphenol A diglycidyl ethers (BADGEs) and novolac glycidyl ethers (NOGEs) in marine mammals from the United States coastal waters. Environ. Sci. Technol. 50, 1703–1710.
Xue, J., Venkatesan, A.K., Wu, Q., Halden, R.U., Kannan, K., 2015. Occurrence of bisphenol a diglycidyl ethers (BADGEs) and novolac glycidyl ethers (NOGEs) in archived biosolids from the U.S. EPA's targeted national sewage sludge survey. Environ. Sci. Technol. 49, 6538–6544.
Xue, J., Wan, Y., Kannan, K., 2016. Occurrence of bisphenols, bisphenol A diglycidyl ethers (BADGEs), and novolac glycidyl ethers (NOGEs) in indoor air from Albany, New York, USA, and its implications for inhalation exposure. Chemosphere 151, 1–8.
Xue, J., Liu, W., Kannan, K., 2017. Bisphenols, benzophenones, and bisphenol A diglycidyl ethers in textiles and infant clothing. Environ. Sci. Technol. 51, 5279–5286.
Zhang, L., Wang, F., Ji, Y., Jiao, J., Zou, D., Liu, L., Shan, C., Bai, Z., Sun, Z., 2014. Phthalate esters (PAEs) in indoor PM10/PM2.5 and human exposure to PAEs via inhalation of indoor air in Tianjin, China. Atmos. Environ. 85, 139–146.