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ORIGINAL ARTICLE

Selected bisphenols and phthalates screened for estrogen and androgen disruption by *in silico* and *in vitro* methods

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Abstract

OBJECTIVES: The aim of this study was to detect endocrine disruption potential of selected bisphenols and phthalates, compare *in silico* prediction with results from two *in vitro* methods and bring up-to-date information on development of EU legislation, available *in vitro* methods and biomechanisms involved in endocrine disruption.

MATERIAL AND METHODS: *In silico* approach based on the OECD QSAR Toolbox was used for prediction of estrogen receptor α binding. OECD TG 455 assay and a yeast-based YES/YAS assay was used to determine the interactions with human estrogen (ERα) and androgen receptors.

RESULTS: *In silico* results predicted the screened phthalates as non binders and bisphenols as very strong binders of the ERα. *In vitro* results differed from *in silico* prediction in several cases but exhibited concordance mainly for strong binders of ERα. Most of the substances exhibited parallel activity (agonist-antagonist) on both estrogen and androgen receptors. Agonistic studies showed the effective concentration of 10% activity (EC_{10}) from 5.0E-07 for strong agonists (e.g. BPC, BPTMC). Cytotoxicity was observed after 48 h exposure of *S. cerevisiae* to BPFL, BPG, BPM, BPTMC in concentrations starting at 3.6E-05 mol/l.

CONCLUSION: Our results suggest multiple parallel interactions of tested compounds and emphasize the importance of determination of an appropriate battery of in vitro methods that will include more receptors and will be appropriate to target specific molecular mechanisms involved in endocrine disruption. Results in agonistic studies indicate agonistic potential and are supported by results of antagonistic studies with consideration of possible multiple interactions.

Abbreviations:

ADME	- Absorption, Distribution, Metabolism, Excretion
AR	- androgen receptor
BBP	- benzyl butyl phthalate
BPBP	- bisphenol BP
BPC	- bisphenol C
BPFL	- bisphenol FL
BPG	- bisphenol G
BPM	- bisphenol M
BPP	- bisphenol P
BPTMC	- bisphenol TMC
CF	- Conceptual Framework
DEP	- diethyl phthalate
DEHP	- bis(2-ethylhexyl) phthalate
DBP	- dibutyl phthalate
DHT	- 5α-dihydrotestosterone

DIBP - diisobutyl phthalate
DINP - diisononyl phthalate
DIDP - diisodecyl phthalate
E2 - 17β-estradiol
EDs - endocrine disruptors
ER - estrogen receptor
GD - Guideline Document
MW - molecular weight
EFSA - European Food Safety Authority
ECHA - European Chemicals Agency
JRC - the Joint Research Centre
OD - optical density
OECD - Organization for Economic Cooperation and Development
OPPTS - Office of Prevention, Pesticides & Toxic Substances
QSAR - Quantitative Structure-Activity Relationship
TG - Test Guideline
US EPA - United States Environmental Protection Agency

INTRODUCTION

Endocrine disruptors (EDs) are exogenous ligands capable to bind to cellular receptors or serum transport proteins, potentially contributing to signaling pathways modulation, endocrine system disturbances and consequent developmental, reproductive and system disorders (Latchney *et al.* 2018; Frye *et al.* 2012; Marty *et al.* 2010; Skah *et al.* 2017). Sources of exposure come from industry or agriculture, including consumer products, e.g. food packaging materials, thermopaper, plastics, paintings, household products or cosmetics. Interaction of ligands with receptors is a molecular initiation event that leads to complex effects. The physiological receptor mechanism may be affected either by direct receptor binding, resulting in activation (agonistic activity) or inhibition (antagonistic activity), or consequent modulation of associated signaling pathways´ regulation. Human receptors may share ligands including endocrine disruptors with varying selectivity, affinity and efficacy, of which certain may be persistent, leading to bioaccumulation, while others may be rapidly metabolised and act for a limited time (Balaguer *et al.* 2017; Farman & Rafestin-Oblin 2001; Hothersall *et al.* 2016; Wang *et al.* 2016; Wagner *et al.* 2001). Certain endogenous ligands are hydrophilic molecules unable to pass through the plasma membrane such as glycoproteins (e.g., thyroid stimulating hormone, follicle–stimulating hormone, luteinizing hormone), catecholamines (e.g., dopamine, adrenaline) and peptide hormones (e.g., prolactin, somatotropin, adrenocorticotropic, antidiuretic, parathyroid hormone, calcitonin, oxytocin, insulin, glucagon) with target transmembrane receptors. Receptors for lipophilic endogenous ligands able to enter the cell via the plasma membrane such as steroid (e.g., estrogen, testosterone, progesterone), thyroid (triiodothyronine, thyroxine) and corticosteroid (cortisol, corticosterone, cortisone, aldosterone) hormones are located in the cytoplasm, functioning as transcription factors (Schweizer *et al.* 2014; Yang *et al.* 2015). The organism may be exposed to a mixture of chemically diverse potential ligands with variable affinity, efficacy and resistance time, e.g., bisphenols, phthalates, parabens, alkylphenols, polyaromic hydrocarbons, polychlorinated and polybrominated biphenyls, perfluoralkyls, pesticides, organotins, synthetic hormones etc., as well as natural compounds such as mycotoxins or phytoestrogens (Diamanti-Kandarakis et al. 2009; Sifakis *et al.*, 2017). Physiological effects *in vivo* may be influenced by various factors, such as developmental stage, bioavailability, distribution, metabolic transformation, tissue disposition and elimination (Bruning *et al.* 2016; Liu *et al.* 2015; Kato *et al.* 2006). Human exposure may be influenced also by individual physiological, medical, social and ecological factors (e.g., health condition, medication, health disorders, biorhythm, aging, individual variability of metabolism, genetic mutations and polymorphism, nutrition, environment, smoking, stress), that may modulate number of receptors, kinetics, bioaccumulation, and cause synergic and additive interactions (Lovallo *et al.* 2015, Brody *et al.* 2014, Pivovarova *et al.* 2012, Lambert *et al.* 2004, Folmer 2018; Dahl & Akerud 2013). With regard to the above mentioned biomechanisms and factors, it is difficult to attribute identified endocrine disruption *in vivo* to a distinct endocrine disruptor, as the associations of ED´s levels with selected biomarkers of action (e.g. hormone, enzyme or protein levels) may not directly confirm causal relationships in case of such multifactorial exposure (Vineis & Kriebel 2006). Development and use of *in silico* screening tools and *in vitro* methods is therefore effective for first-level primary screening and should be used more intensively for hazard identification. Numerous biological *in vitro* methods based on transfected cell lines or yeast have been recently developed. OECD Test Guidelines (TGs) and standardized test methods are listed in the OECD Guidance document No. 150 (OECD, 2012) for evaluating chemicals for endocrine disruption. The OECD GD 150 was updated (Update v3) in December 2017 and describes new assays of all levels (Level 1–5) included in the updated OECD Conceptual Framework (CF) for Testing and Assessment of Endocrine Disrupting Chemicals. Read across, chemical categories, QSAR and other *in silico* and ADME model predictions may be used at Level 1. TGs provide data about physical and chemical properties, e.g. MW, reactivity, volatility, biodegradability. At Level 2, TGs provide *in vitro* data about selected endocrine mechanisms and pathways, covering mammalian and non mammalian test systems. For Level 3–5 only *in vivo* assays are available, using various models (insects, crustaceans, gastropods, amphibians, fish, rodents, avians) (OECD 2012).

Test Guidelines for *in vitro* determination of various endpoints of endocrine disruption listed in OECD GD 150, available for Level 2 of OECD CF, comprise e.g. the following endpoints:

- Estrogen or androgen receptor binding affinity (OECD TG 493), (US EPA TG OPPTS 890.1150)
- Estrogen receptor transactivation (OECD TG 455)
- Androgen receptor transactivation (OECD TG 458)
- Steroidogenesis *in vitro* (OECD TG 456)
- Aromatase assay (US EPA TG OPPTS 890.1200)
- Thyroid disruption assays (e.g. thyroperoxidase inhibition, transthyretin binding)
- Retinoid receptor transactivation assays
- Other hormone receptors assays as appropriate
- High-throughput screens (OECD GD No. 211 Describing Non-Guideline In Vitro Test Methods)

In our continuous pilot study, selected bisphenols and phthalates were tested using OECD QSAR Toolbox for *in silico* prediction of estrogenic potential. Two *in vitro* methods based on human cell line and yeast were used in order to determine the interactions of the tested chemicals with human estrogen and androgen receptors.

MATERIAL AND METHODS

Selected phthalates and bisphenols, i.e. Diethyl phthalate (DEP), Bis(2-ethylhexyl) phthalate (DEHP), Benzyl butyl phthalate (BBP), Dibutyl phthalate (DBP), Diisobutyl phthalate (DIBP), Diisononyl phthalate (DINP), Diisodecyl phthalate (DIDP), Bisphenol BP (BPBP), Bisphenol C (BPC), Bisphenol FL (BPFL), Bisphenol G (BPG), Bisphenol M (BPM), Bisphenol P (BPP), Bisphenol TMC (BPTMC) (Sigma Aldrich) were tested in a continuous pilot study for endocrine activity, compared to relevant analytical standards (Methoxychlor, 17β-estradiol – E2, 4-Hydroxytamoxifen, 5α-dihydrotestosterone – DHT, Flutamide). Chemical structures of the tested compounds are indicated in Table 1.

OECD QSAR toolbox

OECD QSAR Toolbox (Toolbox 3.3.2 Release Notes) was used for prediction of potential ligands and their binding affinity to the estrogen receptor α based on their chemical structure, molecular weight and partition coefficient octanol-water. For ER binding endpoint, OECD QSAR Toolbox contains categories of ER binders and is relevant for reproductive toxicity endpoints in fish and mammals. The ER-binding profiler classifies chemicals as non binders or binders depending on molecular weight (MW) and structural characteristics of the chemicals: very strong binders (chemicals with MW between 200 and 500 Da and two rings with a hydroxyl group connected to each of them), strong binders (chemicals with at least one 5- or 6-members carbon ring with an unhindered hydroxyl or amino group and MW between 200 and 500 Da), moderate binders (chemicals with at least one 5- or 6-members carbon ring with an unhindered hydroxyl or amino group and MW between 170 and 200 Da), weak binders (chemicals with at least one 5-or 6-members carbon ring with an unhindered hydroxyl or amino group and MW less than 170 Da). If the chemical does not meet the structural and parametric requirements, it is classified as non binder, e.g. non binder with impaired hydroxyl or amino group, non binder with MW more than 500 Da, non binders without hydroxyl or amino group; non-cyclic non binder.

Stably transfected transactivation in vitro assay to detect estrogen receptor agonists (OECD TG 455)

A continuous human cell line VM7Luc4E2, graciously provided by Prof. Michael Denison, UC Davis, California, USA, for research purposes during the initial part of the study, was used for confirmation of OECD QSAR Toolbox prediction. The assay is based on binding of a tested substance to ERα. The culture and assay was performed according to recommended procedure, with minor modifications (Rogers & Denison 2000). In brief, cells were cultured in MEMα medium (Gibco), containing 10% fetal bovine serum and 1% penicillin/streptomycin. Five days prior analysis, cells were cultured in Dulbecco´s modified eagle medium, estrogen stripped and phenol red free (Sigma Aldrich), with 8% charcoal stripped fetal bovine serum and 1.9% supplement of L-glutamine with daily media change. Cells were plated in 96-well plates (100 µl per well) at a concentration of 500.000 cells/ml and incubated for 24 h (37 °C, 5% CO_2). The next day the plated cells were treated with tested compounds in triplicates in selected concentrations for 24 h (37 °C, 5% CO_2). Luciferase Assay System (Promega) in combination with Glomax Multi Plus Injector Luminometer (Promega) was used for luminiscence measurement of the ERα activation by the test substance.

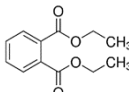
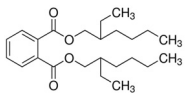
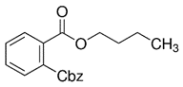
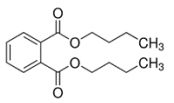
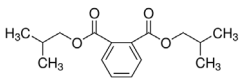
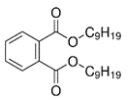
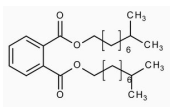
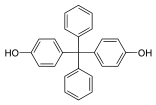
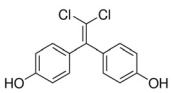
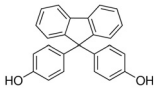
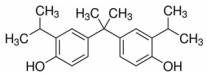
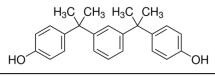
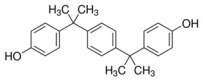
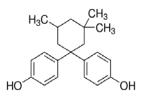
Yeast based reporter gene assay (Xenoscreen YES/YAS)

A commercially available yeast-based microplate assay (Xenoscreen YES/YAS, Xenometrix®, Switzerland) designed for detection of compounds with estrogenic and androgenic agonistic/antagonistic activities of chemicals, water samples and biological fluids, based on recombinant *Saccharomyces cerevisiae* strains with human estrogen (hERα) and androgen (hAR) receptors was used as a comparative test to OECD TG 455 method and for determination of interactions of the tested compounds with human androgen receptor. The assay was performed according to the provided standard operating procedure, using the supplied standardized material and chemicals. Briefly, the precultured cell suspension was exposed to the tested compounds for 48 hours on orbital shaker. The OD of the red product resulting from conversion of the yellow substrate after secretion of β-galactosidase was measured on Biotec Eon™ High Performance Microplate Spectrophotometer at 570 nm. The OD_{570} of the end product in comparison with controls provides direct correlation with the endocrine activity of the tested substances.

RESULTS

Structure and MW of the tested compounds are listed in Table 1. *In silico* and *in vitro* results are presented in Table 2. Using the OECD QSAR Toolbox, a compound was categorized as a strong binder (+++) according to MW (between 200 and 500 Da), chemical structure (two rings with a hydroxyl group connected to each of them) and partition coefficient octanol-water. If the compound did not meet structural and parametric requirements, it was predicted as non binder (N) (chemicals with impaired hydroxyl or amino group or without hydroxyl or amino group). *In silico* results predicted all the screened phthalates as non binders and

Tab. 1. Chemical structures of tested phthalates and bisphenols.

Compound	CAS No.	M.W.	Chemical formula	Structural formula
Diethyl phthalate (DEP)	84-66-2	222.24	C6H4-1,2-(CO2C2H5)2	
Bis(2-ethylhexyl) phthalate (DEHP)	117-81-7	390.56	C24H38O4	
Benzyl butyl phthalate (BBP)	85-68-7	312.36	2-[CH3(CH2)3O2C] C6H4CO2CH2C6H5	
Dibutyl phthalate (DBP)	84-74-2	278.34	C6H4-1,2-[CO2(CH2)3CH3]2	
Diisobutyl phthalate (DIBP)	84-69-5	278.34	C6H4-1,2-[CO2CH2CH(CH3)2]2	
Diisononyl phthalate (DINP)	28553-12-0	418.61	C6H4(CO2C9H19)2	
Diisodecyl phthalate (DIDP)	26761-40-0	446.66	C28H46O4	
Bisphenol BP (BPBP)	1844-01-5	352.43	C25H20O2	
Bisphenol C (BPC)	79-97-0	256.34	(CH3)2C[C6H3(CH3)OH]2	
Bisphenol FL (BPFL)	3236-71-3	350.41	C25H18O2	
Bisphenol G (BPG)	127-54-8	312.45	C21H28O2	
Bisphenol M (BPM)	13595-25-0	346.46	C6H4[C(CH3)2C6H4OH]2	
Bisphenol P (BPP)	2167-51-3	346.46	C6H4[C(CH3)2C6H4OH]2	
Bisphenol TMC (BPTMC)	129188-99-4	310.43	C21H26O2	

all the screened bisphenols as very strong binders to ERα, as indicated in Table 2. The results of agonistic *in vitro* assays (indicated in Table 2) were categorized as follows: the result categorized as +++ (strong) indicates that the substance (BPC, BPTMC, BBP) showed a strong concentration-dependent response with a concentration response curve consisting of a baseline, followed by a slope concluding in a plateau or peak, while the difference between the baseline and peak of the highest non-cytotoxic concentration was at least 70% of the maximal value for the positive control (17β-estradiol in agonistic assay with the ERα receptor, 5α-dihydrotestosterone in agonistic assay with the AR receptor). The result categorized as ++ (moderate) indicates that the substance (BBP, BPG, BPM, DBP) showed a concentration-response curve consisting of a baseline, followed by a slope concluding in a plateau or peak, while the difference between the baseline and peak of the highest non-cytotoxic concentration was at least 40% of the maximal value for the positive control. The result categorized as + (weak) indicates that the substance (DBP, BPG, BPFL) showed a weak response, consisting of a baseline, followed by a slope or peak, and the difference between the baseline and peak of the highest non-cytotoxic concentration was at least 20% of the maximal value for the positive control. The result categorized as N (negative) indicates that the substance did not show a response consisting of a baseline, a slope or peak in non-cytotoxic concentrations (DINP, DIDP, DEP, DEHP, BPBP, BPFL) or showed a weaker response than + (weak). The results of antagonistic *in vitro* assays (indicated in Table 2) were categorized as follows: the result categorized as +++ (strong) indicates that the substance (BPP, BPC, BPM) showed a strong concentration-dependent response with a declining curve consisting of a baseline, followed by a decline, while the lowest value in non-cytotoxic concentration was not higher than 130% of the lowest value for the positive control (4-hydroxytamoxifen in the antagonistic assay with the ERα receptor, Flutamide in the antagonistic assay with the AR receptor). The result categorized as ++ (moderate) indicates that the substance (BPBP, BPG, BPP, BPTMC, DBP) showed a concentration-dependent response with a declining curve consisting of a baseline, followed by a decline, while the lowest value in non-cytotoxic concentration was not higher than 200% of the lowest value for the positive control. The result categorized as + (weak) indicates that the substance (BBP, DBP, DEHP, DIBP, BPBP, BPFL, BPG, BPM) showed a weak concentration-dependent response with a declining curve consisting of a baseline, followed by a decline, while the lowest value of non-cytotoxic concentration was not higher than 300% of the lowest value for the positive control. The result categorized as N (negative) indicates that the substance (DIDP, DINP, BPFL, BPC) did not show a concentration-dependent response with a declining curve consisting of a baseline, followed by a decline, and the lowest value of non-cytotoxic concentration was higher than 300% of the lowest value for the positive control. Cytotoxicity was observed after 48 h exposure of *S. cerevisiae* to BPFL, BPG, BPM, BPTMC in concentrations starting at 3.6×10^{-5} mol/l, as indicated in Table 2. Effective concentration EC_{10} (the concentration that causes the measured effect in 10% of cells) of selected bisphenols achieved values of 5.0E-07 for strong agonists (e.g. BPC), as indicated in Table 3.

Tab. 2. Endocrine activity of tested compounds.

Substance	ER binding (QSAR)	ER agonist (OECD 455)	ER agonist (YES)	ER antagonist (YES)	AR agonist (YAS)	AR antagonist (YAS)
Diethyl phthalate (DEP)	N	N	N	N	N	N
Bis(2-ethylhexyl) phthalate (DEHP)	N	N	N	N	N	+
Benzyl butyl phthalate (BBP)	N	+++	++	N	N	+
Dibutyl phthalate (DBP)	N	++	N	++	N	+
Diisobutyl phthalate (DIBP)	N	++	N	N	N	+
Diisononyl phthalate (DINP)	N	N	N	N	N	N
Diisodecyl phthalate (DIDP)	N	N	N	N	N	N
Bisphenol BP (BPBP)	+++	N	N	+	N	++
Bisphenol C (BPC)	+++	+++	+++	N	N	+++
Bisphenol FL (BPFL)*	+++	N	+	+	++	N
Bisphenol G (BPG)*	+++	+	++	+	N	++
Bisphenol M (BPM)*	+++	++	+	+	N	+++
Bisphenol P (BPP)	+++	++	N	+++	N	++
Bisphenol TMC (BPTMC)*	+++	+++	+++	N	N	++

*In the highest possible tested concentration: 10^{-5} mol/l; +++ - strong binder; ++ - moderate binder; + - weak binder; N – non binder

Tab. 3. Effective concentration (EC_{10}) of selected bisphenols in YES/YAS agonist assays.

YES Agonist Assay	EC_{10}
BPC	5.0E-07
BPTMC	4.0E-06
BPFL	8.8E-06
BPG	1.9E-05
E2 (Positive control)	5.3E-11
YAS Agonist Assay	**EC_{10}**
BPFL	4.9E-06
DHT (Positive control)	5.1E-10

DISCUSSION

Our *in vitro* results differed slightly from *in silico* prediction and exhibited good concordance regarding the estrogenic activity, mainly for strong binders. OECD QSAR Toolbox prediction correlated with *in vitro* results in negative prediction for DEP, DEHP, DIDP, DINP, and differed in case of BBP, DBP and DIBP that showed estrogenic potential *in vitro*. QSAR prediction of categorization differed from *in vitro* results in case of BPBP, BPFL, BPG, BPM, BPP, predicted as very strong binders, while *in vitro* estrogenic potential was detected as moderate or weak. Our results show that *in silico* prediction should be confirmed by *in vitro* results in various biological systems, however, the OECD QSAR Toolbox turns out to be a promising starting point for prediction of chemical groups with possible endocrine potential. For the activity of compounds against the androgen receptor (AR) the YAS method was used and the correlation of results with an appropriate *in silico* tool would be interesting study in the future. Unfortunately, there are still few *in silico* models with AR binding endpoint internationally validated and accepted. In antagonist assays targeting the mechanism of inhibition of agonist binding to the receptor, the binding of the agonist and antagonist is generally presumed to be mutually exclusive. However, several types of agonism and antagonism have been described recently, providing information about possible multiple activities of substances and their interactions with the receptors. For example, agonist-antagonists may show both agonist and antagonist properties, which may be the case of most of the tested substances. Specific molecular reactions may occur, e.g. the substance may bind to non-specific recognition site on the receptor (allosteric agonist), dissociate (reversible antagonist) or form stable chemical bonds (irreversible antagonists), etc. In competitive antagonism, binding of an antagonist should prevent binding of the agonist, but in case of noncompetitive antagonism, agonist and antagonist can be bound simultaneously, whereas the antagonist reduces the action of the agonist. In case of reversible competitive antagonism, agonist and antagonist form short-lasting bonds with the receptor, reaching a steady state. *In vivo*, a substance that acts as a (partial) agonist in one tissue may act as a (full) agonist in another. Substances may be also nonspecific binders and bind to molecular sites e.g. on serum proteins (in blood *in vivo* or in media or reagents used *in vitro*), preventing the transport of endogenous hormones. Biochemical mechanisms should be considered when evaluating the results *in vitro* (and even more *in vivo*), taking into account the precautionary principle (Salahudeen & Nishtala 2017; Allegretti *et al.* 2016; Bookout *et al.* 2006; Yang *et al.* 2006, Lambert 2004). Our results confirm the above mentioned multiple interactions of the substances with the receptors and suggest agonistic studies to be more reliable when supported by results from antagonistic studies, in order to detect the overall parallel interactions of the substances to the estrogen and androgen receptors. Both agonistic and antagonistic studies should support the overall evaluation of the substance as a binder or non-binder, whereas variability of the results in various biological systems may be observed beside the above mentioned biochemical mechanisms, e.g. due to inhibition of cell wall transport to the yeast cell or cytotoxicity in higher concentrations. It is advisable to monitor viability, use multiple concentrations of positive controls and samples and final non-cytotoxic concentration of solvents (e.g. 1% DMSO), all of which was carefully monitored in our study. The complexity in biological mechanisms of endocrine disruption emphasizes the importance of further development of an appropriate battery of tests that will include more receptors (such as thyroid, retinoid, aryl hydrocarbon, peroxisome proliferator-activated receptor, liver X, vitamin D, pregnane X, growth hormone receptor, etc.), enabling the detection of more specific endocrine activities, involved in e.g. reproduction and development, steroidogenesis, metabolism, energy homeostasis, central nervous system regulation, etc. (Bookout *et al.* 2006, Yang *et al.* 2006). Numerous *in vitro* methods have already proved scientific relevance and have become publicly available (OECD 2012). *In vitro* methods based on yeast have already been included in ISO standards (ISO 2017) and are used for (eco)toxicological purposes. With regard to recent developments in the EU legislation, the onset of increasing pressure in the near future can be expected for testing chemicals mainly from the group of plant protection products.

From 10 November 2018 (EC 2018b), a substance shall be considered as having endocrine disrupting properties, if (1) it shows an adverse effect in non-target organisms, (2) it has an endocrine mode of action; (3) the adverse effect is a consequence of the endocrine mode of action. The identification of an active substance as having endocrine disrupting properties that may cause adverse effect in humans shall be based on: all available relevant scientific data (*in vivo* studies or

adequately validated alternative test systems predictive of adverse effects in humans or animals; as well as *in vivo*, *in vitro*, or, if applicable, *in silico* studies informing about endocrine modes of action (EC 2018a). Guidance on identifying endocrine disruptors was developed and published by scientific staff from European Chemical Agency (ECHA) and the European Food Safety Authority (EFSA), with the support of the Joint Research Centre (JRC) to ensure harmonised implementation of the endocrine disruptor criteria throughout the EU for the assessment of biocides and plant protection products. The Guideline advises applicants and assessors of the competent regulatory authorities on how to identify endocrine disruptors in accordance with the criteria. The criteria for biocides apply from 7 June 2018. (ECHA & EFSA, 2018; EC, 2018a).

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