Using assessment criteria for pesticides to evaluate the endocrine disrupting potential of non-pesticide chemicals: Case butylparaben

Boberg, Julie; Johansson, Hanna Katarina Lilith; Axelstad Petersen, Marta; Olsen, Gustav Peder Mohr; Johansen, Mathias; Holmboe, Stine A.; Andersson, Anna-Maria; Svingen, Terje

Published in:
Environment International

Link to article, DOI:
10.1016/j.envint.2020.105996

Publication date:
2020

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Review article

Using assessment criteria for pesticides to evaluate the endocrine disrupting potential of non-pesticide chemicals: Case butylparaben

Julie Boberg⁎, Hanna K.L. Johansson⁎, Marta Axelstad⁎, Gustav P.M. Olsen⁎, Mathias Johansen⁎, Stine A. Holmboe, Anna-Maria Andersson⁎, Terje Svingen

⁎Division of Diet, Disease Prevention and Toxicology, National Food Institute, Technical University of Denmark, Kongens Lyngby, Denmark
⁎Department of Growth and Reproduction and International Centre for Research and Research Training in Endocrine Disruption of Male Reproduction and Child Health (EDMaRC), Rigshospitalet, University of Copenhagen, Copenhagen, Denmark

A R T I C L E   I N F O
Handling Editor: Olga-Ioanna Kalantzis
Keywords:
Risk assessment
Endocrine disruptors
EDC
Parabens
Test guideline
Butyl 4-hydroxybenzoate

A B S T R A C T
Regulation of chemicals with endocrine disrupting properties depend on the use of the chemical rather than its intrinsic properties. Within the EU, the only criteria currently in place for identifying an endocrine disrupting chemical (EDC) are those developed for biocidal and plant protection products. We argue that ECHA/EFSA guidance for assessing endocrine disrupting properties of biocidal and plant protection products can be applied to all chemicals independent of their intended use. We have assessed the REACH-registered compound butyl paraben (CAS 94-36-8), a preservative used primarily in cosmetics. Based on scientific evidence of adverse reproductive effects and endocrine activity, the open literature suggest that butylparaben is an EDC. By applying the ECHA/EFSA guidance for pesticides and biocides, we identify butylparaben as a compound with endocrine disrupting properties. Even though available data is markedly different from that for biocides and pesticides, it was possible to reach this conclusion. Moreover, we propose that the ECHA/EFSA guidance can and should be used for identification of EDC regardless of their intended application.

1. Introduction

In 2018, the EU Commission published criteria to evaluate endocrine disrupting properties of biocidal and plant protection products (herein referred to as pesticides) with the aim to reduce human exposure to endocrine disrupting chemicals (EDCs). All biocides and pesticides need, by law, to be assessed for their potential endocrine disrupting properties. To aid in the assessment, the new guidance documents set down by the European Food Safety Authority (EFSA) and the European Chemicals Agency (ECHA) (ECHA/EFSA et al., 2018) provides additional guidance on how to do this, albeit formally not a law. This EU legislation can be considered an implementation into law of the WHO/IPCS definition of an EDC: "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations" (WHO/IPCS, 2002). Notably, however, these new EU criteria are limited to biocides and pesticides. This, we believe, could potentially allow many non-biocide/pesticide chemicals to evade EDC identification. In fact, a recent targeted stakeholder consultation report issued by the European Commission highlights that the vast majority of the responders consider that “the absence of harmonised criteria poses a problem to the identification of endocrine disruptors across sectors” (European Commission, Joint Research Council, 2020).

In the REACH regulation (article 57f), endocrine disruptors may be identified, on a case-by-case basis, as substances of very high concern (SVHC) where there is scientific evidence of probable serious effects to human health or the environment. This leads to an equivalent level of concern as CMR (Carcinogenic, Mutagenic or toxic to Reproduction) or PBT/vPvB (Persistent, Bioaccumulative and Toxic/Very Persistent and Very Bioaccumulative) substances. Identification of substances with endocrine disrupting properties under REACH is also based on the definition of the WHO/IPCS, but no defined guidance for this evaluation of endocrine disrupting properties is provided within REACH. We propose to use the same guidance for identification of endocrine disruption that has been developed for pesticides/biocides for other chemical groups as well. By so doing, we aim to better safeguard the general population against potential effects associated with hormone disruption.

Abbreviations: AGD, anogenital distance; ECHA, European Chemicals Agency; EDC, Endocrine disrupting chemical; EA(T)S, estrogenic, androgenic, (thyroid), steroidogenic; EFSA, European Food Safety Authority; MoA, Mode of Action
⁎ Corresponding author.
E-mail address: jub@food.dtu.dk (J. Boberg).

https://doi.org/10.1016/j.envint.2020.105996
Received 23 April 2020; Received in revised form 30 June 2020; Accepted 16 July 2020
0160-4120/ © 2020 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/BY-NC-ND/4.0/).
The commonality between all EDCs is that they disrupt normal hormone-mediated action and, by so doing, cause adverse effects in an intact organism, including humans, or its progeny. With regard to the reproductive system, the most common effect modalities are disrupted androgen or estrogen signaling, or steroid biosynthesis. There are many different chemical classes that have been identified as EDCs or suspected EDCs, including phthalates, azoles, bisphenols and parabens (ChemSec, 2020; Hass et al., 2018; TEDX, 2020). Notably, some of these suspected EDCs are used in a variety of products and are therefore governed by different legislations depending on the end use. One example of a group of chemicals falling into these different legislations (Danish EPA, 2013), is the parabens.

Parabens are synthetic preservatives commonly used in cosmetics, pharmaceuticals and foods (Nowak et al., 2018). They have been increasingly used as anti-microbial agents since first reported on for their anti-microbial properties back in 1924 (Goddard and McCue, 2001). Human exposure to parabens is widespread and thus a potential health concern (Boberg et al., 2010). Certain parabens have been evaluated as being, or suspected to be endocrine disruptors, namely butylparaben, isobutylparaben, propylparaben, ethylparaben and methylparaben (Hass et al., 2018; Hass et al., 2012a; Hass et al., 2012b). As background documentation to a proposal for classification of butylparaben as a substance of very high concern (SVHC) (ECHA – European Chemicals Agency, 2020), we evaluated the endocrine disrupting properties of butylparaben. Since no criteria or guidance is available for evaluation of endocrine disruption within REACH, we used butylparaben as a case

**Fig. 1.** Flow chart for evaluation of endocrine disrupting effects of chemicals. * Our mode of action evaluation focused specifically on EAS (estrogenic, androgenic, steroidogenic) activities and adverse effects in perinatally exposed males.
to test whether non-pesticides could be identified as EDCs based on the ECHA/EFSA guidance documents set out for biocides and plant protection agents (ECHA/EFSA et al., 2018). Notably, as a result of the SVHC classification proposal butylparaben was recently added to the EU Candidate List of SVHCs (added 26th June 2020), which now comprises 209 substances that may have serious effects on people or the environment (https://echa.europa.eu/candidate-list-table; ECHA – European Chemicals Agency, 2020).

The intention of the 2018 ECHA/EFSA guidance document is to provide applicants and assessors from regulatory authorities with the required information to implement scientific criteria for determining endocrine disrupting properties of biocides and pesticides. In this study, we apply the same approach for a REACH substance in order to provide a systematic overview of the available data. The criteria for identification of biocides and pesticides as endocrine disruptors (European Commission, 2018) are based on WHO/IPCS definitions (WHO/IPCS, 2002). According to these criteria, a substance shall be considered as having endocrine disrupting properties if it meets all of the following criteria:

i) it shows an adverse effect in [an intact organism or its progeny]/ [non-target organisms], which is a change in the morphology, physiology, growth, development, reproduction or life span of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences;

ii) it has an endocrine mode of action, i.e. it alters the function(s) of the endocrine system;

iii) the adverse effect is a consequence of the endocrine mode of action.

To permit endocrine disruption evaluation, the ECHA/EFSA guidance document from 2018 provides comprehensive direction, including stepwise methodology for data collection, lines of evidence and mode of action (MoA) analysis. Herein, we follow this guidance for butylparaben and present data collection, lines of evidence analysis, and MoA analysis for endocrine disrupting properties, before discussing the utility of this approach for REACH compounds falling outside this evaluation framework.

2. Methods

Our evaluation included four steps as shown in Fig. 1. A data collection step led to presentation of data in a ‘lines of evidence’ analysis. Next, a MoA analysis was performed for the selected modalities EAS (estrogenic, androgenic, steroidogenic) and for the selected adverse effects (male reproductive effects of perinatal or pubertal/adult exposure). The MoA analysis included the evaluation of a biologically plausible link between key events, evaluation of dose and temporal concordance, essentiality of key events, human relevance, and uncertainties. This enabled us to conclude whether or not endocrine disruption criteria were met. This manuscript presents an overview of the process, whereas detailed evaluations and descriptions of data collection are found in Supplementary Files 1 to 5.

2.1. Data collection

According to guidance from ECHA/EFSA (2018), an applicant preparing an assessment of a plant protection or biocidal product should include information from standard information requirements, as well as other scientific data selected applying a systematic review methodology. For pesticides, EFSA provides guidance on application of systematic review methodology (EFSA, 2010), and on submission of scientific peer-reviewed open literature (EFSA, 2011), which should be followed for ED assessment of plant protection or biocidal products.

We deviate from the ECHA/EFSA guidance by not using the EFSA 2010 and 2011 guidance. However, we consider our literature search strategy to be in accordance with these principles. For human epidemiological studies, search was carried out in Pubmed using the terms {"butyl paraben" OR butylparaben OR “butyl 4-hydroxybenzoate” OR “butyl p-hydroxybenzoate” AND human}. The search for in vivo and in vitro studies was done in three steps. First, relevant studies up to 2017 were identified in pre-existing, comprehensive literature reviews on butylparaben, including a series of risk assessment reports from the European Scientific Committee for Consumer Safety (SCCS, formerly SCCP) (Boberg et al., 2010; Brand et al., 2017; Hessel et al., 2019; SCCS, 2013; CIR, 2017). Next, this was followed up by a search limited to the period from Jan 2017 to Aug 2019 using search terms "paraben AND (toxicity OR safety)" and "butylparaben", as the comprehensive reports contained overview tables over studies up to 2017. Finally, we selected studies related to endocrine and reproductive targets in vivo and in vitro, which resulted in a list of 47 relevant studies. This outcome is expected to be similar to the outcome of a systematic search using equivalent terms, since the outcome builds on review reports providing systematic literature searches supplied with a broad search for recent studies. We thus consider that following EFSA 2010 and 2011 guidance on data collection will not have influenced the outcome of our data collection.

Scientific literature on the toxicity of butylparaben covers a wide range of target tissues and mechanisms. This includes reproductive and developmental toxicity, endocrine targets, immunotoxicity, and metabolic disorders. To enable endocrine disruption evaluation, we focused our data collection on endocrine and reproductive effects, as these targets were considered of key importance in previous evaluations by Brand et al. (2017) and Hessel et al. (2019). All relevant data from the identified in vivo experimental literature were registered in an excel-based tool developed by ECHA/EFSA, followed by data extraction according to the guidance document and data entry into the accompanying Excel file (ECHA/EFSA et al., 2018) (see Supplementary File 5). For evaluation of epidemiological studies we developed an Excel (Microsoft) template (see Supplementary File 4) for systematic extraction of information on main parameters and estimation of the associated weight according to “study quality considerations for weighting epidemiological observational studies” as defined in EFSA (2017). Endocrine-related adverse effects can be grouped according to estrogenic, androgenic, thyroidal and steroidogenic modalities (EATS) based on the Organisation for Economic Co-operation and Development (OECD) revised guidance document for evaluating chemicals for ED properties (OECD, 2018b) and the Joint Research Centre’s (JRC) screening methodology to identify potential ED compounds (JRC, 2016). Since previous evaluations of butylparaben have not indicated thyroid activity, we did not include thyroid-specific in vitro and in vivo endpoints in the data registration step. For each in vitro assay, the specific endpoints that relate to specific endocrine MoA are indicated. For in vivo tests it could not be determined which endpoints relate to which of the modalities E, A or S, and hence is not stipulated. The results of the literature search and data registration can be found in Supplementary files 3 to 5.

2.2. Lines of evidence analysis

We performed a selection process to assemble lines of evidence from the collated data (‘excel tool’) in accordance with the guidance document (ECHA/EFSA et al., 2018). We narrowed our selection to only include in vitro and in vivo studies related to steroid hormone synthesis and receptor activation, and to mammalian studies on adverse effects on the reproductive system in vivo. These lines of evidence for adverse effects and endocrine activity of butylparaben are found in Supplementary File 1 and provide an overview of available evidence and sources of information.

In vivo evidence, including hormone levels, is generally considered stronger evidence of an endocrine disrupting MoA than in vitro evidence with respect to mechanistic knowledge (JRC, 2016). The rationale for this is that any observed effect that occurs in vivo inherently accounts
for absorption, distribution, metabolism and excretion (ADME) of the chemical, as well as the *in vivo* MoA being more closely linked to the manifestation of the adversity than would be the case *in vitro* (JRC, 2016).

*In vivo* adverse effects can be divided into i) EATS-specific endpoints, ii) non-specific adversity, which account for endpoints that are sensitive to, but not specific of, EATS pathways involvement, and iii) general adversity, which includes systemic toxicity and effects unrelated to EATS pathways. Examples of EATS-mediated effects are shorter anogenital distance (AGD) and increased nipple retention in male offspring, altered puberty timing and reproductive organ weights as well as affected sperm parameters (OECD, 2018b). We included epidemiological data as supportive evidence. As stipulated in (ECHA/EFSA et al., 2018), any available epidemiological studies should be considered, but cannot be used to overrule or dismiss evidence of adversity found in laboratory studies, nor can they replace laboratory studies. According to the same guidance document, a positive conclusion on a) Adversity based on ‘EATS-mediated’ parameters and b) Mechanistic tests at OECD Conceptual Framework level 2/3 (*in vitro* and *in vivo* mechanistic) lead to the next step of performing MoA analysis.

### 2.3. MoA analysis

To conclude that a substance is an EDC based on criteria specified by ECHA/EFSA, it is necessary to identify sufficient strength of evidence of endocrine-related adverse effects as well as endocrine activity. In addition, a plausible biological relationship must exist between an endocrine MoA and the adverse effect based on a weight of evidence approach. A MoA analysis was performed in accordance with the guidance document (ECHA/EFSA et al., 2018). The MoA analysis included evaluation of biological plausibility of the link between key events, evaluation of dose and temporal concordance, essentiality of key events, human relevance, and uncertainties. It is emphasized by ECHA/EFSA that both biological plausibility and empirical support are weighted; however, biological plausibility should be considered as stronger evidence. We carried out MoA analyses for perinatal and adult exposure, but below we present only the analysis for perinatal exposure.

### 3. Results

The outcome of our literature search, lines of evidence analysis and MoA analysis are presented in detail in Supplementary Files 1 to 5. These annexes correspond to supporting documents to the ECHA decision in June 2020 to consider butylparaben an SVHC (ECHA – European Chemicals Agency, 2020).

#### 3.1. Lines of evidence

Our lines of evidence analysis included an overview of data pertaining to endocrine activity *in vitro* and *in vivo*, as well as adverse effects *in vivo*. We focused the analysis on selected molecular mechanisms and adverse effects on the male reproductive system. The evidence regarding endocrine activity is the same regardless of exposure period but, as reflected in the MoA analysis, we have separated *in vivo* evidence into perinatal and pubertal/adult exposure periods (Fig. 2) as the same endocrine activity may result in different adverse effects or different severity of adverse effects depending on period of exposure.

##### 3.1.1. Evidence for endocrine activity from *in vitro* and *in vivo* mechanistic studies

The first step in the lines of evidence analysis is to evaluate the strength of evidence for endocrine activity. We found strongest evidence for estrogenic activity, both *in vitro* and *in vivo* (Table 1 and Supplementary File 1). A subset of studies also suggest steroidogenic or androgenic modalities, but these data were less consistent and thus considered as ‘supporting evidence only’ for endocrine activity.

Some studies have shown that butylparaben can act as an Estrogen receptor (ER) agonist (Gonzalez et al., 2018; Pop et al., 2018; Watanabe et al., 2013), whereas the metabolite 4-hydroxybenzoic acid (PHBA) has lower affinity (reviewed by Boberg et al 2010). Several studies report that butylparaben can induce proliferation of estrogen-sensitive cells (Charles and Darbre, 2013; Gonzalez et al., 2018; Khanna and Darbre, 2013; Pop et al., 2018; Williams and Darbre, 2019) or growth of uterus in the *in vivo* uterotropic bioassay in rodents (Goswami and Kalita, 2016; Hossaini et al., 2000; Lemini et al., 2004; Lemini et al., 2003; Routledge et al., 1998; Vo and Jeung, 2009). However, two uterotropic studies reported no effect (Guerra et al., 2017a; Shaw and DeCatanzaro, 2009). One study showed no effect after 21 days of exposure, but changes in uterine histology (Vo et al., 2010).

One study indicates that butylparaben can affect steroidogenesis by upregulating Cyp19a1 (*aromatase*) expression, increase aromatase enzyme activity, and increase estradiol levels in different cell lines (Williams and Darbre, 2019). Another study reports decreased aromatase activity in microsomes from human placenta after exposure (van Meeuwen et al., 2008). Other studies report no increase in estradiol levels in other cell lines, indicating that the response may be cell type-specific (Guerra et al., 2016; Taxvig et al., 2008; Wröbel and Gregoraszczuk, 2013).

Studies reporting on the potential for butylparaben to have AR-antagonistic activity are inconsistent (Chen et al., 2007; Kjaerstad et al., 2010; Pop et al., 2018; Watanabe et al., 2013). Studies have consistently shown that butylparaben has no AR agonist activity (Chen et al., 2007; Gonzalez et al., 2018; Pop et al., 2018).

#### 3.1.2. Evidence for endocrine activity and adverse effect from *in vivo* studies 3.1.2.1. Perinatal exposure

Lines of evidence analysis of butylparaben revealed supporting evidence for endocrine activity (affected AGD and hormone levels) and strong evidence for adverse effects on sperm parameters with perinatal exposure (Table 1 and Supplementary File 1). Some studies that report effect on rodent female reproductive tissues after perinatal exposure were considered ‘supporting evidence’ for endocrine activity, with a plausible chance that endocrine disrupting effects would manifest in vivo.

Several studies show reduced sperm counts or quality in adult male rats after perinatal exposure (Boberg et al., 2016; Guerra et al., 2017b; Kang et al., 2002; Zhang et al., 2014). One of these studies did not show an effect on sperm count, but rather a reduced number of progressively motile sperm and increased numbers of abnormal sperm (Guerra et al., 2017b). Differences in study design (route and timing of exposure) may explain some of the different effects observed between studies.

Shorter male AGD in rodents is a clear sign of endocrine activity and considered an adverse outcome, as it is a marker for reproductive effects in humans (Schwartz et al., 2019a; OECD, 2013; OECD, 2018a). Available literature indicates that perinatal exposure to butylparaben can affect AGD in male offspring, measured at PD 1, using doses of 400 mg/kg bw/day or higher (Boberg et al., 2016; Zhang et al., 2014). A shorter male AGD was not observed at the same doses in late gestation (Taxvig et al., 2008) or at PD 1 in studies administering lower doses of butylparaben (Guerra et al., 2017b; Kang et al., 2002). Serum hormone levels are reported to be affected across the studies, but with variable effects. Most studies show lower testosterone and higher estradiol levels, but with either higher or lower LH and FSH levels depending on study and age (Guerra et al., 2017b; Zhang et al., 2016; Zhang et al., 2014).

Indications of effects in female rodents following in *utero* or early postnatal exposure to butylparaben were considered insufficient evidence of adverse effects, but supportive of endocrine activity of butylparaben. In brief, ovarian and uterine effects were seen in female Sprague-Dawley rats exposed from PND 1 to 7 by subcutaneous injections to butylparaben (Ahn et al., 2012). A second study, exposing Holzman rat dams from GD 6 to PND 21 by subcutaneous injections,
reported delayed puberty, altered hormone levels, reduced estrous cycle length and altered ovarian and uterine histology (Maske et al., 2018). A third study, exposing Wistar rats orally from GD 7 to PND 22, reported reduced female AGD, reduced ovary weights and altered mammary outgrowth (Boberg et al., 2016). Collectively, clear evidence of adverse reproductive effects were strongest in males, whereas supporting evidence of endocrine activity was seen in both males and females.

3.1.2.2. Adult exposure. For pubertal or adult exposure, the lines of evidence analysis revealed supporting evidence for endocrine activity in vivo (altered hormone levels) and supporting evidence for adverse effects (decreased sperm count, decreased number of normal sperm cells and altered testicular histopathology) (Table 1 and Supplementary File 1). Reduced sperm numbers were reported in three smaller studies (Garcia et al., 2017; Oishi, 2001; Riad et al., 2018), but not in a larger industry-sponsored study (Hoberman et al., 2008). The study by Hoberman et al (2008) failed to corroborate a previous study, which had reported reduced sperm numbers (Oishi, 2001). However, based on shortcomings in the Hoberman et al (2008) study, the European Union Scientific Committee on Consumer Safety (SCCS, 2013) concluded that the ‘no effect’ study by Hoberman et al (2008) was not sufficient to refute Oishi (2001) findings. More recently, two studies have reported reduced sperm numbers in butylparaben-exposed rats (Garcia et al., 2017; Riad et al., 2018), but data on sperm motility and morphology were inconsistent between the studies. Overall, there is evidence for altered sperm parameters in some, but not all studies. In line with SCCS (2013), we cannot use these studies to reach a firm conclusion, and thus we regard the evidence as insufficient of adverse effects with pubertal/adult exposure.

In adult males, altered levels of testosterone, LH and FSH were seen at selected ages after 3–10 weeks of dosing, but were not consistent between studies or ages at examination (Riad et al., 2018; Hoberman et al., 2008; Oishi, 2001; Oishi, 2002; Riad et al., 2018). These data suggest that endocrine activity differs depending on study design. A limited number of studies have examined effects in female rodents exposed to butylparaben during adulthood. The study findings were not considered sufficient evidence of adverse effects, but considered supportive of endocrine activity and biological plausibility that the effects can lead to adverse effects in vivo. In adult female Sprague-Dawley rats exposed for 5 weeks, estrous cycle length and ovarian histology and gene expression was affected (Lee et al., 2017), and another study showed increased estradiol levels at 6–10 h after a single dose of butylparaben (Pollock et al., 2017).

3.1.3. Evidence for endocrine activity and adverse effect from in human studies

We did not identify any epidemiological studies examining the association between intrauterine butylparaben exposure and effects on reproductive parameters (hormone levels and sperm parameters) in men. A few epidemiological studies have examined the relationship between butylparaben exposure in men and reproductive parameters (hormone levels and sperm parameters) (see Supplementary File 4). In one study, an inverse association was seen between male urinary...
<table>
<thead>
<tr>
<th>Title</th>
<th>Hypothesis</th>
<th>Brief description of event</th>
<th>Supporting evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation of ER to Impaired fertility of male offspring</td>
<td>The molecular initiating event is activation of the ER(s). In developing males, increased ER signaling results in altered testicular development in offspring and subsequently altered testicular function in adulthood. In turn, reduced sperm count and quality is observed in offspring.</td>
<td>Molecular: Activation of ER</td>
<td>High. Lines of evidence show sufficient evidence for endocrine activity related to estrogen receptor activation. Several studies show estrogen receptor agonistic response similar to estrogen (Gonzalez et al., 2018; Pop et al., 2018; Wannabe et al., 2013).</td>
</tr>
<tr>
<td>Altered steroidogenesis/increased estradiol level to Impaired fertility of male offspring</td>
<td>The molecular initiating event is altered steroidogenesis/increased estradiol level. In developing males, this leads to increased estrogen receptor (ER) signaling resulting in altered testicular development in offspring and subsequently altered testicular function in adulthood. In turn, reduced sperm count and quality is observed in offspring.</td>
<td>Molecular: Altered steroidogenesis/increased estradiol level</td>
<td>Moderate. Lines of evidence show supporting evidence for endocrine activity related to altered steroidogenesis. One study shows upregulation of aromatase/Cyp19a1 gene expression and increased aromatase enzyme activity as well as increased estradiol levels in different cell lines (Williams and Darbre, 2019). Other studies show no increase of estradiol levels in other cell lines, and the response may be cell type specific (Guerra et al., 2016; Taxvig et al., 2018; Weiβel and Gregoraszczuk, 2013).</td>
</tr>
<tr>
<td>AR antagonism to Impaired fertility of male offspring</td>
<td>The molecular initiating event is antagonism of the AR. In developing males, reduced androgen receptor signaling results in altered testicular development in offspring and subsequently altered testicular function in adulthood. In turn, reduced sperm count and quality is observed in offspring.</td>
<td>Molecular: AR antagonism</td>
<td>Moderate. Lines of evidence show supporting evidence for endocrine activity related to AR antagonism. Studies on AR-antagonistic activity show inconsistent findings. Effect in some (Chen et al., 2007; Pop et al., 2018), but not all studies (Kjaerntal et al., 2010; Wannabe et al., 2013), possibly due to different study design. Studies on AR-activation consistently show that butylparaben has no AR agonist activity (Chen et al., 2007; Gonzalez et al., 2018; Pop et al., 2018).</td>
</tr>
</tbody>
</table>

| KE1 Increased ER signaling | Molecular: Increased ER signaling | High. Several studies show effects on growth of estrogen sensitive cells (Charles and Darbre, 2013; Gonzalez et al., 2018; Khanan and Darbre, 2013; Pop et al., 2018; Williams and Darbre, 2019) or tissues (uterotrophic assay in vivo (Goyaneni and Kalita, 2016; Hossaini et al., 2000; Lemini et al., 2004; Lemini et al., 2003; Roughedge et al., 1998; Vo and Jeung, 2009)). | Moderate. |

| KE2 Organ: Altered reproductive development of male offspring | Organ: Altered reproductive development of male offspring | Moderate. Reduced AGD in males at PND 1 and 21 (Boberg et al., 2018; Zhang et al., 2014), but other studies showed no effect on AGD at PND 1 (Guerra et al., 2017b; Kang et al., 2002) or in males GD 21 (Taxvig et al., 2008). Inconsistency between studies on AGD may be due to different exposure periods, dose levels and measuring sensitivity. The two studies including doses of 400 mg/kg bw/day or above both showed reduced sperm counts at these doses (Boberg et al., 2016; Zhang et al., 2014). A dose of 100 mg/kg bw/day reduced AGD in one study (Boberg et al., 2016), but in other studies doses in the same range (10–200 mg/kg bw/day) did not affect AGD (Guerra et al., 2017b; Kang et al., 2002; Zhang et al., 2014). No changes in fetal testis histology (Boberg et al., 2016; Guerra et al., 2017b). Signs of histological effects on seminiferous tubules of prepubertal testes in one study (Zhang et al., 2014). | Moderate. |

| KE3 Organ: Altered testicular and epididymal function of adult offspring | Organ: Altered testicular and epididymal function of adult offspring | Moderate. Altered serum levels of T, E2 (and LH, FSH; increase or decrease depending on study design) (Guerra et al., 2017b; Zhang et al., 2017). No reports of change in epididymal histology. | Moderate. |

| AO1 Organ: Reduced sperm count and quality of offspring | Organ: Reduced sperm count and quality of offspring | High. All studies using perinatal exposure caused altered sperm count and/or quality, though different parameters were affected in different studies. Reduced epididymal sperm count (50–75% of control; (Boberg et al., 2016; Kang et al., 2002)) but no change in epididymal sperm count in another study (Guerra et al., 2017b). Reduced sperm motility (60% of control, (Kang et al., 2002)) and reduced percentage of progressive motile sperm (low dose only, (Guerra et al., 2017b)). Increased percentage of sperm with head abnormalities and reduced percentage of normal sperm (Guerra et al., 2017b). | Low. |

| AO2 Organism: Impaired fertility of male offspring | Organism: Impaired fertility of male offspring | Limited evidence for effect in rodents, but high plausibility that impaired sperm count and quality in humans lead to impaired fertility. No effect on fertility assessed by natural mating or artificial insemination (Guerra et al., 2017b). | Low. |
butylparaben levels and sperm concentration and motility (Smarr et al., 2018), whereas three studies observed no significant associations between male urinary butylparaben and classical sperm parameters (Joensen et al., 2018; Meeker et al., 2011; Nishihama et al., 2017). The study reporting effects on sperm parameters was considered more reliable than the three negative studies (Supplementary File 4), and is considered as supporting evidence for adverse effects of butylparaben exposure in adulthood. In women, supporting evidence for endocrine effects was seen in a study reporting a negative association between maternal urinary butylparaben levels and maternal serum levels of oestradiol and the oestradiol/progesterone ratio (Aker et al., 2019).

As described by ECHA/EFSA (2018), available epidemiological studies need to be considered, but cannot be used to override or dismiss evidence of adversity found in laboratory studies, nor to replace laboratory studies (ECHA/EFSA, 2018).

3.1.4. Evaluation of evidence of endocrine activity and adverse effect

According to the ECHA/EFSA (2018) guidance, positive conclusions on a) Adversity based on ‘EATS-mediated’ parameters and b) Mechanistic tests at OECD Conceptual Framework level 2/3 (in vitro and in vivo) mechanistic lead to the next step of performing MoA analysis. Our lines of evidence analysis of butylparaben revealed sufficient evidence for endocrine activity and sufficient evidence for adverse effects on sperm parameters with perinatal exposure (Supplementary File 1). No further data on MoA, or effects of butylparaben, are considered necessary.

For perinatal and adult exposure, no conclusion was reached with regard to adverse effect, and according to ECHA/EFSA (2018) this leads to the next step of performing a MoA analysis. However, we conclude that not all of the ‘EAS-mediated’ parameters in relation to adverse effects have been investigated, meaning that additional studies addressing these knowledge gaps must be performed to reach a sound conclusion.

3.2. MoA analysis

Here, we present only the MoA analysis for effects of perinatal exposure, although the same process was carried out for pubertal/adult exposure. The steps in the MoA analysis included an overview of key events (Fig. 2), an analysis of biological plausibility of key event relationships, and considerations on dose and temporal concordance, human relevance and uncertainties.

For perinatal exposure, the analysis led us to conclude that butylparaben acts via multiple MoAs, and it is biologically plausible that the endocrine activities of butylparaben lead to the observed adverse effects on the male reproductive system following perinatal exposure (Table 2 and 3 and Supplementary File 2).

For ER activation, directly or by increased oestradiol levels, the evidence for each key event relationship was considered “High”, except the step “Increased estrogen receptor signaling” (KE1) to “Altered reproductive development of offspring” (KE2), for which the evidence was considered “Moderate to high”. For AR antagonism, evidence for all key event relationships were considered “High”. There was sufficient dose and temporal concordance between key events, and the effects are assumed relevant for humans (Supplementary File 2). Overall, the analysis led to the conclusion that butylparaben acts via multiple MoAs, and it is biologically plausible that the endocrine activities of butylparaben can lead to the observed adverse effects on the male reproductive system following perinatal exposure. There were no indications from the scientific literature of alternative non-endocrine MoAs being operative.

As the MoA of butylparaben is based on “EATS mediated adversity” and the evidence for adverse effect of perinatal exposure is considered sufficient, the criteria for endocrine disrupters are considered met.

4. Discussion

We have shown that, by applying the EFSA/ECHA guidance for determining if a biocide or pesticide have endocrine disrupting properties, the REACH registered compound butylparaben would be considered an EDC. It is our evaluation that the tools developed for evaluation of biocides and plant protection products are useful for this purpose, albeit with some limitations as discussed below.

4.1. Use of ECHA/EFSA guidance

The principles of assessment of endocrine disruption for biocides/pesticides (ECHA/EFSA, 2018) is considered useful for other chemical groups, and this evaluation of butylparaben serves as an excellent example of a data-rich compound. Other conclusions might be reached with data-poor substances. For REACH chemicals, data availability differs markedly, and for e.g. food additives the testing requirements may not include sufficient information on endocrine sensitive endpoints. For some substances where in vivo data or human epidemiological data are lacking, it may be impossible to reach conclusions on adverse effect. This may be the case for certain substances for use in cosmetic products where animal testing is prohibited. The guidance and excel-based tool for data collection is considered useful, but limitations were identified, particularly with regard to entering data from non-guideline studies. More specifically, the excel-based tool builds on the principle that it continuously narrows down the available options so that the result description can be as specific as possible. This is a strength when using guideline studies; however, it can be a limitation when working with the open literature, especially in vitro studies. The reason for this is that the first selection when entering data is ’Type of toxicity’ and ’Study principle’. With these categories, it can be challenging to include literature concerning in vitro studies. In our experience, most of the ‘in vitro studies’ were placed under a general category. This becomes problematic when reaching the step of generating lines of evidence, as the studies then are not classified according to EAS modalities. For this reason, we conducted the EAS classification manually for the in vitro studies.

4.2. Evaluation of endocrine disruption for butylparaben

We conclude that there is sufficient evidence of endocrine activity (ER activation, and possibly altered steroidogenesis and AR antagonism) and adverse effect for butylparaben. The reported adverse health effects include reduced sperm count and quality in rodent studies. These effects are considered severe, since similar effects in humans could cause infertility. The effects are considered irreversible and manifest late in life following exposure during perinatal life. This evaluation served as background documentation to the SVHC classification proposal (ECHA – European Chemicals Agency, 2020). This has different consequences depending on the use of the substance.

Importantly, this evaluation is purely a hazard assessment, and risk is not considered in an ED evaluation (ECHA/EFSA, 2018). Nevertheless, the obtained data overview provides information useful for risk assessment purposes. No safe dose (concentration) can be derived from the available data on adverse reproductive effects via endocrine MoA. Two of the available studies show reduced sperm count or quality in perinatally-exposed rats at the lowest tested dose of 10 mg/kg bw/day with oral and subcutaneous exposure, respectively (Boberg et al., 2016; Guerra et al., 2017b), and no NOAEL can be determined for this endpoint. The most recent risk assessment by the EU Scientific Committee on Consumer Safety (SCCS, 2013) was based on another study which did not include evaluation of these endpoints (Fisher et al., 1999). Considering the abundance of new toxicity data for butylparaben since 2013, and a potential change in use of butylparaben, an update of risk assessment may be warranted.
### Table 2
Analysis of biological plausibility of key event relationships.

<table>
<thead>
<tr>
<th>Title</th>
<th>Brief description of key event relationship (KER)</th>
<th>Supporting evidence</th>
<th>Brief description of key event relationship (KER)</th>
<th>Supporting evidence</th>
<th>Brief description of key event relationship (KER)</th>
<th>Supporting evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIE to KE1</td>
<td>Estrogen receptor activation to Increased transcription of estrogen receptor regulated genes</td>
<td>High. Estrogen receptor activation leads to increased estrogen receptors signaling.</td>
<td>Altered steroidogenesis/increased estradiol level to Impaired fertility of male offspring</td>
<td>High. Altered steroidogenesis/estradiol level to Increased estrogen receptor signaling</td>
<td>Androgen receptor antagonism to Reduced androgen receptor signaling</td>
<td>High. Antagonism of androgen receptor leads to reduced androgen receptor signaling.</td>
</tr>
<tr>
<td>KE1 to KE2</td>
<td>Increased estrogen receptor signaling to Altered reproductive development of offspring</td>
<td>Moderate to high. ERα is expressed in fetal Leydig cells (Nielsen et al., 2000) and has regulatory effects on steroidogenesis; endogenous estrogens inhibit testicular development and function in fetal/early neonatal life (Delbès et al., 2005; Delbès et al., 2006). Exogenous 'estrogens' lead to decreased testosterone levels in rodents (Delbès et al., 2005; Delbès et al., 2004; Lussegrue et al., 2003; Lehni et al., 2013). In turn, reduced testosterone levels in male fetuses may cause masculinization failure (Schwartz et al., 2019c; Stewart et al., 2018).</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KE2 to KE3</td>
<td>Altered reproductive development of offspring to Altered testicular and epididymal development in adult offspring</td>
<td>High. Correct development of the reproductive system in early life is essential to achieve optimal reproductive function in adulthood. It is highly biologically plausible that impaired reproductive development is a cause of altered testicular and epididymal function in adulthood.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KE3 to AO1</td>
<td>Altered testicular and epididymal development in adult offspring to Reduced sperm count and quality in offspring</td>
<td>High. Correct function of testes and epididymis is necessary for an optimal sperm count and quality (motility, morphology)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AO1 to AO2</td>
<td>Reduced sperm count and quality in offspring to Impaired fertility of male offspring</td>
<td>High. There is clear evidence that impaired sperm count and quality in humans leads to impaired fertility. In rodents, reproductive function is less sensitive to reductions in sperm count and quality.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3
Result from the Mode of action analysis (Supplementary File 2).

<table>
<thead>
<tr>
<th>Mode of action analysis</th>
<th>There is sufficient evidence of endocrine activity (ER activation and possibly altered steroidogenesis and AR antagonism) and adverse effect (decreased sperm count and quality).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biological plausibility</td>
<td>It is biologically plausible that adverse effects are due to the endocrine activity of butylparaben.</td>
</tr>
<tr>
<td>Dose and temporal concordance</td>
<td>In each study, indicators of key events related to endocrine activity are affected at the same doses causing adverse effects. Between studies, there are differences in effective doses. Key events are observed in the hypothesized order, i.e. in vivo indicators of endocrine activity are seen in developing animals, and adverse effects are seen in adulthood.</td>
</tr>
<tr>
<td>Essentiality</td>
<td>Essentiality has not been investigated.</td>
</tr>
<tr>
<td>Human relevance</td>
<td>Human relevance is assumed, as there are no data indicating that these endocrine modes-of-action are not relevant to humans.</td>
</tr>
<tr>
<td>Uncertainties</td>
<td>This uncertainty analysis highlights that the evidence base for butylparaben is relatively limited, yet there is consistency between different studies on endocrine mode of action and adverse effects.</td>
</tr>
</tbody>
</table>

Conclusion: Butylparaben acts via multiple modes of action and it is biologically plausible that the endocrine activities of butylparabens lead to the observed adverse effects on the male reproductive system. For ER activation (directly or due to increased estradiol levels) the evidence for each key event relationship is considered “High”, except for the step “Increased ER signaling to Altered reproductive development of offspring”, for which the evidence is considered “Moderate to high”. For AR antagonism, evidence for all key event relationships is considered “High”. There is sufficient dose and temporal concordance between key events, and effects are assumed relevant to humans.

5. Conclusions

We conclude that butylparaben is an EDC according to criteria developed for evaluating biocides and pesticides (ECHA/EFSA et al., 2018). We base this conclusion on clear evidence for endocrine MoA and adverse effects as well as a biologically plausible link between the two. Specifically, we found strong evidence for butylparaben exerting an estrogenic MoA both in vitro and in vivo, but also evidence supporting both steroidogenic and androgenic modalities. The strongest evidence for adverse effects is altered sperm parameters following perinatal exposure, with supportive evidence given from observations of altered male AGD or histological changes in testes in a subset of studies.

With respect to the use of the ECHA/EFSA guidance to chemicals not categorized as biocides or pesticides, we consider it a very useful tool, albeit in need of some optimization. In particular, the result will depend on data availability, and the entry of in vitro data needs further refinement, either by expanding existing categories or by adding a new category at the data entry point. Regardless, we would support further efforts towards harmonizing criteria used for classifying chemicals as EDCs and move away from the current situation where classifications are delayed simply because different chemicals end up in different legislative silos based on intended application rather than inherent properties.

CRediT authorship contribution statement

Julie Boberg: Conceptualization, Methodology, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition. Hanna K.L. Johansson: Methodology, Formal analysis, Data curation, Writing - review & editing. Marta Axelstam: Funding acquisition, Conceptualization, Methodology, Formal analysis, Writing - review & editing. Gustav P.M. Olsen: Data curation, Writing - review & editing. Mathias Johansen: Data curation, Writing - review & editing. Stine A. Holmboe: Formal analysis, Data curation, Writing - review & editing. Anna-Maria Andersson: Formal analysis, Writing - review & editing. Terje Svingen: Conceptualization, Writing - original draft, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

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

Acknowledgements

We like to thank Anders Rehfeld, Hanne Frederiksen and Louise Scheutz Henriksen for their additional evaluations of studies. We also thank Sofie Christiansen and Rune Hjorth for critical reading and helpful comments on the manuscript.

Funding

Danish Environmental Protection Agency.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105996.

References

ECHA/EFSA, European Chemical Agency (ECHA) and European Food Safety Authority (EFSA) with the technical support of the Joint Research Centre (JRC), Anderson, N., Arena, M., Auteri, D., Barmaez, S., Grigera, E., Kiemler, A., Lepper, P., Lostia, A.M., Munn, S., Morto, J.M.P., Pellizzato, F., Tarazona, J., Terron, A., Van der Linden, S., 2018. ECHA (European Chemicals Agency) and EFSA (European Food Safety Authority) with the technical support of the Joint Research Centre (JRC). Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No
biomarkers of antimicrobial exposure and bi-directional associations with semen
Stewart, M.K., Mattiske, D.M., Paik, A.J., 2018. In utero exposure to both high- and low-
dose diethylstilbestrol disrupts mouse genital tubercle development. Biol. Reprod. 99,
1184–1193.
Tan, K.A., De Genst, K., Atanassova, N., Walker, M., Sharpe, R.M., Saunders, P.T.,
Denolet, E., Verhoeven, G., 2005. The role of androgens in sertoli cell proliferation and
functional maturation: studies in mice with total or Sertoli cell-selective ablation
of the androgen receptor. Endocrinology 146, 2674–2683.
Taxvig, C., Vinggaard, A.M., Hass, U., Axelstad, M., Boberg, J., Hansen, P.R., Frederiksen,
H., Nellermann, C., 2008. Do parabens have the ability to interfere with ster-
endocrinedisruption.org/interactive-tools/tedx-list-of-potential-endocrine-
disruptors/search-the-tedx-list.
Aromatase inhibiting and combined estrogenic effects of parabens and estrogenic
Vo, T.T., Yoo, Y.M., Choi, K.C., Jeung, E.B., 2010. Potential estrogenic effect(s) of para-
bens at the prepubertal stage of a postnatal female rat model. Reprod. Toxicol. 29,
306–316.
Comparative study on transcriptional activity of 17 parabens mediated by estrogen
receptor α and β and androgen receptor. Food Chem. Toxicol. 57, 227–234.
Williams, G.P., Darbre, P.D., 2019. Low-dose environmental endocrine disruptors, in-
crease aromatase activity, estradiol biosynthesis and cell proliferation in human
Wróbel, A., Gregoraszczuk, E.L., 2013. Effects of single and repeated in vitro exposure of
three forms of parabens, methyl-, butyl- and propylparabens on the proliferation and
Zhang, L., Ding, S., Qiao, P., Dong, L., Yu, M., Wang, C., Zhang, M., Zhang, L., Li, Y., Tang,
N., Chang, B., 2016. n- butylparaben induces male reproductive disorders via reg-
Zhang, L., Dong, L., Ding, S., Qiao, P., Wang, C., Zhang, M., Zhang, L., Du, Q., Li, Y., Tang,
N., Chang, B., 2014. Effects of n-butylparaben on steroidogenesis and spermatogen-
esis through changed E2 levels in male rat offspring’. Environ. Toxicol. Pharmacol.
37, 705–717.