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Birgit Mertens[1], C. Simon[2], M. Van Bossuyt[1], Matthias Onghena[3], T. Vandermarken[4], K. Van Langenhove[4], H. Demaegdt[5], E. Van Hoeck[1], J. Van Loco[1], K. Vandermeiren[5], Adrian Covaci[3], M.L. Scippo[2], M. Elskens[4] and L. Verschaeve[1,6]*

[1]*Department of Food, Medicines and Consumer Safety, Scientific Institute of Public Health (Site Elsene), J. Wytsmanstraat 14, Brussels, Belgium*

[2]*Departement of Food Science, University of Liège - FARAH-Veterinary Public Health, Quartier Vallée 2, Avenue de Cureghem 10, Sart Tilman B43bis – Liège, Belgium*

[3]*Toxicological Center, Department of Pharmaceutical Sciences, University of Antwerp, Universiteitplein 1, Wilrijk, Belgium*

[4]*Department of Analytical, Environmental and Geo-Chemistry, Vrije Universiteit Brussel, Bd de la plaine 2, Ixelles, Belgium*

[5]*CODA-CERVA, Department of Chemical safety of the food chain, Leuvensesteenweg, 17, Tervuren, Belgium*

[6]*Department of Biomedical Sciences, University of Antwerp, Universiteitsplein 1, Wilrijk, Belgium*

[*] To whom correspondence should be addressed. E-mail: **birgit.mertens@wiv-isp.be**, phone: +32 2 642 54 40, fax: +32 2 642 52 24

Abstract (200 words)

Bisphenol A (BPA), a starting material in the manufacture of epoxy resins and polycarbonate (PC) plastics, has been reported to migrate into food. Due to the worldwide concern that BPA might act as an endocrine disruptor, alternative materials for PC have been introduced on the European market. However, PC-replacement products might also release substances of which the toxicological profile – including their genotoxic effects - has not yet been characterized. Because a thorough characterization of the genotoxic profile of the large number of these substances is impossible in the short term, a strategy was developed in order to prioritize those substances for which additional data are urgently needed. The strategy consisted of a decision tree using hazard information related to genotoxicity. The relevant information was obtained from the database of the European Chemicals Agency (ECHA), *in silico* prediction tools (ToxTree and Derek Nexus™) and the *in vitro* Vitotox® test for detecting DNA damage. By applying the decision tree, substances could be classified into different groups, each characterized by a different probability to induce genotoxic effects. For all substances investigated in the current study, more genotoxicity data are needed, but the type and the urge for these data differs among the substances.

Keywords (6)

DNA damage; Vitotox®; *in silico*; ECHA database; food contact materials; polycarbonate replacement products

List of abbreviations

BaP: benzo[a]pyrene; BPA: bisphenol A; CLP: Classification, Labelling and Packaging; ECHA: European Chemicals Agency; 4-NQO: Nitroquinoline 1-oxide; PC: polycarbonate; REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals; SA: structural alert; S/N: signal to noise ratio.

1. Introduction

Bisphenol A (BPA) is often used as a starting material to manufacture epoxy resins and polycarbonate (PC) plastics. Polycarbonates, a group of transparent thermoplastic polymers, have many applications including the fabrication of some food contact materials (FCMs), like infant feeding (baby) bottles, cups, etc (EFSA, 2015). Reports on the migration of BPA from PC into food together with studies identifying BPA as an endocrine disruptor have resulted in a worldwide concern about the application of BPA in FCMs (Alonso-Magdalena et al., 2012; Nam et al., 2010; Palanza et al., 2008; Talsness et al., 2009). In 2011, the European Commission decided to prohibit the use of BPA in the manufacture of PC baby bottles in the European Union on the basis of the precautionary principle (European Union, 2011a). As a result of this decision, a wide variety of alternative materials for PC has been introduced on the European market. Examples include, amongst others, polypropylene, polyamide, polyethersulphone and a co-polyester under the trade name Tritan^{TM}, but also non-plastics, such as silicone (Onghena et al., 2014; Simoneau et al., 2012). However, BPA-free polymers might also release substances of which the toxicological profile has not yet been (completely) characterized. These migration products include (i) residual starting products due to incomplete polymerisation, (ii) additives that are not chemically linked to the polymeric structure and (iii) products resulting from degradation of the polymer (Bittner et al., 2014). In Europe, substances used as monomer or additive in plastic baby bottles should be in accordance with commission regulation (EU) No 10/2011 on plastic materials and articles intended to come into contact with food. Consequently, only substances included in the European Union positive list (Annex I) of the regulation can be used and migration should be below the specific migration limit, if available (European Union, 2011b). In contrast, no specific regulation exists for non-intentionally added substances migrating from plastics (e.g. degradation and reaction products with unknown chemical identity) or substances migrating from non-plastic FCMs, such as silicones. In 2012, Simoneau et al. reported on the migration of substances not included in the EU positive list from baby bottles used as substitutes for PC

(Simoneau et al., 2012). Recently, migration of substances not authorised by the EU legislation for plastic FCMs from PC-replacement baby bottles was confirmed by Onghena et al. (2014 and 2015). More data on the human exposure to and the toxicological properties of these substances are urgently needed to evaluate the toxicity and risks associated with PC-replacement products.

Genotoxicity is an important toxicological endpoint as genetic alterations in somatic and germ cells have been associated with serious health effects including cancer, degenerative diseases, reduced fertility and inherited diseases (Erickson, 2010; Hoeijmakers, 2009; Kong et al., 2012). Consequently, results of genotoxicity tests are key elements in risk assessment of chemicals in general, including those present in food and feed (EFSA, 2011). Also, for substances intended to be used as starting product or additive in plastic FCMs in the EU, genotoxicity data are required, regardless the level of migration (Barlow, 2009). A battery of three *in vitro* genotoxicity tests should be performed including (i) a gene mutation test in bacteria; (ii) an *in vitro* mammalian cell gene mutation test and (iii) an *in vitro* mammalian chromosome aberration test. If any of these tests yields a positive or equivocal result, further genotoxicity tests, including *in vivo* assays, may be required to elucidate the genotoxic potential of the substance (EFSA, 2008). Substances known to be genotoxic are only allowed for use in plastic FCMs under the condition that they do not migrate into food in amounts that are detectable by an agreed sensitive method. In practice, concentrations in food should be below 10 µg/kg (Barlow, 2009).

Considering the large number of substances that can migrate from different types of baby bottles, a complete characterization of the genotoxic profile of all substances is not feasible in the short term. Within this context, a strategy was developed in order to identify chemicals of high concern among the substances for which migration from baby bottles has been reported in literature (Onghena et al., 2014 and 2015; Simoneau et al., 2012). The strategy consisted of a decision tree based on hazard information related to genotoxicity retrieved from literature

combined with results of *in silico* and *in vitro* methods. Firstly, the database of the European Chemicals Agency (ECHA) was consulted to collect data of previous *in vitro* and *in vivo* genotoxicity tests on the selected compounds. Secondly, the genotoxic potential of these substances was predicted by two *in silico* rule-based programmes, i.e. ToxTree and Derek Nexus™. Thirdly, an *in vitro* screening study on their genotoxicity was performed with the Vitotox® test. Because this rapid indicator test uses only small amounts of the test compound for detecting DNA damage, the test was particularly suited for the present study (Westerink et al., 2009). Finally, all information was combined according to a decision tree in order to classify the substances into three groups, each characterized by a different probability to induce genotoxic effects. Substances included in Annex I of the European Regulation 10/2011 were not considered in the present study as these substances have already been subject to evaluation.

2. Materials and methods

2.1. Chemicals

The positive control substances for the Vitotox® test, i.e. benzo[a]pyrene (BaP) and 4-nitroquinoline 1-oxide (4-NQO), were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). An overview of the 48 substances selected for the current study based on the data from Simoneau et al. (2012) and Onghena et al. (2014 and 2015) and their provider is presented in Table 1.

2.2. Data collection from the ECHA database

The ECHA website was consulted in order to collect information on the genotoxic potential of the substances available within the context of the REACH regulation (Registration, Evaluation, Authorisation and Restriction of Chemicals) (European Union, 2006). According to this regulation, the chemical industry must identify and manage the risks linked to the substances they manufacture and market in the EU in the quantity of 1 ton or more per year.

In addition, they have to demonstrate to ECHA how the substance can be safely used, and they must communicate the risk management measures to the users. ECHA has to make the information available so that the general public can make informed decisions about their use of chemicals. For this reason, all information on the substances is collected in an ECHA database which can be consulted on the ECHA website (ECHA, 2015). The information collected from the ECHA database within the present study is summarized below.

2.2.1. In vitro and in vivo genotoxicity data for registered substances

Registration of the chemicals under REACH was checked by introducing the CAS number of each compound in the ECHA database. If registered, toxicological information is publicly available in the database. The type and the amount however depend on the quantity and the use of the substance that is produced or imported. Genotoxicity data (i.e. at least the results of a bacterial gene mutation test) are required for all chemicals produced or imported in quantities of more than 1 ton/year.

The available *in vitro* and *in vivo* data were collected for the different genotoxic endpoints (i.e gene mutations, chromosome damage and non-specific genotoxicity). Only data of studies with a Klimisch score of 1 (reliable without restrictions) or 2 (reliable with restrictions) were retained (Klimisch et al., 1997). A final call for each of the endpoints was made based on the available data. In case one of the *in vitro* gene mutation tests was positive, the substance was considered to induce gene mutations *in vitro*. A similar strategy was applied for the other *in vitro* and *in vivo* endpoints (i.e. *in vivo* gene mutations, *in vitro* and *in vivo* chromosomal damage and *in vitro* and *in vivo* non-specific genotoxicity). Substances were classified as 'clearly genotoxic' in case a positive result was observed in one of the *in vivo* genotoxicity tests. If all *in vitro* studies were negative or *in vitro* positive results were not confirmed in an adequate *in vivo* follow-up test, substances were considered 'not genotoxic'. For substances with insufficient or inadequate data, no final conclusion on the genotoxic potential was formulated.

Although the ECHA database comprises an important amount of data, information should be interpreted with caution as the data are introduced by the registrant. ECHA may examine any registration dossier to verify if the information submitted by registrants is compliant with the legal requirements, but these compliance checks are only required for 5% of the registration dossiers of each tonnage band. Additionally, an evaluation of certain substances is performed by the European Member States in order to clarify whether their use poses a risk to human health or the environment. Only for these substances, a thorough and critical study of the provided toxicity data is performed. Consequently, for most of the substances, the data provided by the registrant have not been evaluated independently. For each of the substances, it was therefore indicated whether or not such an evaluation has been done, is currently on-going or planned in the near future.

2.2.2. Harmonized classification according to the CLP Regulation

Another EU regulation related to chemicals focusses on Classification, Labelling and Packaging (CLP) of substances and mixtures (European Union, 2008). For the present study, information on the harmonized classification of chemicals is most relevant. In order to obtain a harmonized classification of a substance (or mixture), a dossier needs to be introduced containing information on the manufacture and uses of the substances, its hazards and a justification that action is needed at Community level. Member States as well as manufacturers, importers and downstream users may propose the classification and labelling of a substance to be harmonised across the European Union. The information included in the dossier needs to be sufficient to make an independent assessment of various physical, toxicological and ecotoxicological hazards. In the current study, it was investigated whether a harmonized classification for mutagenicity exists for each of the 48 chemicals. Indeed, a harmonized classification of the substance for mutagenicity indicates that based on the available evidence the substance can be considered as mutagenic. A more detailed description

of the different (sub)categories of mutagens according to the CLP Regulation (EC) No 1272/2008 is provided in Table 2.

2.3. *In silico* prediction of genotoxic potential

Two rule-based systems were used, i.e. ToxTree version 2.6.0 and Derek Nexus™ (Lhasa Limited, Leeds, UK) version 4.1.0. In ToxTree, an open-source rule-based system, the Carcinogenicity (genotox and nongenotox) and mutagenicity rulebase by Instituto Superiore di Sanità (ISS) was selected to detect structural alerts (SA) for the endpoint 'genotoxic carcinogenicity'. An outcome of 'Yes' was considered as a positive prediction, whereas the outcome of 'No' was regarded as negative. Negative predictions should however be interpreted with caution. Indeed, negative predictions do not necessarily mean that the compound is not genotoxic; it may be non-genotoxic but it may also be outside the model's domain. For Derek Nexus™, a commercially available expert rule-based system, 'mutagenicity' was selected as an endpoint for the prediction of the potential to induce gene mutations. For this endpoint, the Derek Nexus™ software automatically indicated whether or not the compound was correctly classified. Only for substances without misclassified and unclassified features, the Derek Nexus™ prediction was considered valid. 'Chromosome damage' was used to detect SAs for structural and numerical chromosome aberrations whereas 'non-specific genotoxicity' was used as an endpoint for the prediction of DNA damage in general. Negative predictions for the endpoints other than 'mutagenicity' should, like the negative predictions with ToxTree, be interpreted with caution, as compounds may be outside the model's domain. Compounds were considered to have a SA for the selected endpoint if the prediction in Derek Nexus™ was 'certain', 'probable', 'plausible' or 'equivocal'. The predictions 'doubted', 'improbable', 'impossible', 'inactive' or 'no alert' were regarded as negative.

2.4. Vitotox® test

The Vitotox® test (Gentaur, Kampenhout, Belgium) is a bacterial reporter assay in *Salmonella typhimurium* based on SOS-induction. It uses two different constructs, as described in detail in Verschaeve et al. (1999). One contains a luciferase gene under the control of the recN promoter which results in light production when DNA is damaged (TA104-recN2–4 strain; Genox strain). In the second strain, a luciferase gene under the control of a constitutive promoter is inserted so that the light production is not influenced by genotoxic compounds. This strain (TA104-pr1 strain; Cytox strain) is used as an internal control to detect cytotoxicity and direct interference with bioluminescence.

In brief, Genox and Cytox *Salmonella typhimurium* bacteria were cultivated in 96-well plates and exposed to the test substances. For each of the 48 substances, a dilution series was prepared starting from a 100 mM stock solution in DMSO. In a first experiment, the final concentrations of the test substance in the measurement plate corresponded to 10 µM, 100 µM and 1 mM (in culture medium containing 1% of DMSO). Substances with a low solubility in DMSO were tested at lower concentrations or dissolved in ethanol instead of DMSO. Based on the results of the first experiment and the solubility of the test substance, concentrations for a second experiment were selected. For each substance, minimum five concentrations were tested in at least two independent experiments. Genotoxicity was studied both in the presence and the absence of metabolizing S9 fraction. 4-Nitroquinoline oxide (4-NQO) was used as a positive control without S9 metabolic activation, while benzo[a]pyrene (BaP) was used as the positive control requiring S9 metabolic activation. The final concentrations of 4-NQO and BaP in the measurement plate were 0.020 µM and 32 µM, respectively. Light emission was recorded every 5 minutes after the addition of the test substances to the bacteria, during 4 h using a luminometer (GloMax®, Promega Benelux b.v., Leiden, Nederland). The signal to noise ratio (S/N) or, specifically, the light production of exposed bacteria divided by the light production of non-exposed bacteria, was automatically calculated for each measurement. S/N was calculated for both strains separately. A substance was considered genotoxic when:

-max S/N Genox / max S/N Cytox >1.5

-max S/N in Genox shows a good dose-effect relationship

-max (S/N Genox) / (S/N Cytox) shows a good dose-effect relationship

The test substances were not considered genotoxic if S/N (Genox) increased rapidly within the first 30 min or immediately showed a high value as SOS induction is not yet possible within this short period of time. Test substances were considered cytotoxic when S/N (Cytox) ratios were consistently below 0.8 (Verschaeve et al., 1999).

2.5. Prioritization of substances based on the need for more data

Based on the information from the ECHA database, the *in silico* predictions with ToxTree and Derek Nexus™ and the *in vitro* results of the Vitotox® test, the substances were divided in three main groups according to the decision tree presented in Figure 1.

- Group 1: Substances that can be considered as genotoxic. For these substances, migration from the baby bottles needs to be further investigated in order to evaluate whether concentrations in food are below the threshold of 10 µg/kg.
- Group 2: Substances for which more data to investigate the genotoxic potential are needed.
- Group 3: Non-genotoxic substances for which no further data on genotoxicity are required.

Substances of group 2 were further divided into four subgroups depending on the urgency of the need for further genotoxicity data (Figure 1). Indeed, substances for which there are indications for genotoxicity from *in silico* and/or *in vitro* experiments were prioritized compared to substances for which such indications were lacking.

- Subgroup 2A: very high priority for more genotoxicity data
- Subgroup 2B: high priority for more genotoxicity data
- Subgroup 2C: medium priority for more genotoxicity data
- Subgroup 2D: low priority for more genotoxicity data

3. Results

For the 48 substances selected based on the publications of Simoneau et al. (2012) and Onghena et al. (2014 and 2015), information was obtained from the ECHA database, *in silico* prediction tools (ToxTree and Derek Nexus™) and the *in vitro* Vitotox® test for detecting DNA damage. Next, all information was combined according to a decision tree in order to identify substances of high concern.

3.1. Evaluation of the genotoxic potential based on the information from the ECHA database

Twenty-two out of the 48 substances have been registered under REACH, and genotoxicity data were found in the ECHA database (Table 3). Based on the criteria described under 2.2.1., a final call on the genotoxic potential of the substances was made. None of the substances was identified as clearly genotoxic. For five compounds, i.e. cyclohexanone, dicyclopentyl(dimethoxy)silane, 2-ethylhexyl acrylate, isophorone and 2,4,6-trimethylbenzaldehyde, the available data were equivocal or insufficient and did therefore not allow to conclude on their genotoxic potential. The remaining 17 substances were considered to be non-genotoxic based on the available data. Although an independent evaluation by an European member state is currently on-going for some substances (2-ethylhexyl acetate and isophorone) or planned in the near future (2,4-di-*tert*-butylphenol, naphthalene and octyl methoxycinnamate), no evaluation report is currently available for any of the 22 compounds and consequently, results should be interpreted with caution.

None of the 48 substances has so far been classified as mutagens cat 1A, cat 1B or cat 2 according to the CLP-Regulation (EC) No 1272/2008.

3.2. In silico prediction of genotoxicity with ToxTree and Derek Nexus™

3.2.1. In silico prediction of genotoxicity for the registered substances

Out of the 22 registered substances for which data were available in the ECHA database, 18 did not show a SA for any of the genotoxic endpoints, neither in ToxTree or Derek Nexus™ (Table 4). The four remaining substances displayed SA(s) for at least one of the selected endpoints in ToxTree or Derek Nexus™. However, for three out of these four substances, predictions with the two *in silico* programmes were clearly different and only one substance, i.e. isophorone, triggered both a SA for 'genotoxic carcinogenicity' and 'chromosome damage' in ToxTree and Derek Nexus™, respectively. The substance 2,4,6-trimethylbenzaldehyde showed a SA for 'genotoxic carcinogenicity' only in ToxTree, whereas 2-ethylhexyl acrylate and naphthalene were both predicted to induce 'chromosomal damage' but only with Derek Nexus™.

3.2.2. In silico prediction of genotoxicity for the unregistered substances

Fourteen out of the 26 substances with no information available in the ECHA database, did not show a SA for any of the genotoxic endpoints in ToxTree or Derek Nexus™ (Table 5). Again, important differences were observed between the predictions made by the two *in silico* programmes. Only two substances, i.e. 3,5-di-*tert*-butylbenzoquinone and 2,6-di(*tert*-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one, triggered SAs for genotoxicity in both ToxTree ('genotoxic carcinogenicity') and Derek Nexus™ ('chromosome damage'). Interestingly, all seven substances that triggered a SA for 'genotoxic carcinogenicity' in ToxTree but not for any of the genotoxicity-related endpoints in Derek Nexus™, were benzaldehydes while the three substances that were predicted to induce chromosome damage by Derek Nexus™ but that were not identified as 'genotoxic carcinogens' by ToxTree were all naphthalenes.

3.3. *In vitro* genotoxicity screening with the Vitotox® test

All 48 substances were analysed in minimum two independent experiments with the Vitotox® test. In all experiments performed, the positive control substances 4-NQO (without S9) and BaP (with S9) induced a clear increase in the S/N ratio compared to the negative control in the Genox strain. No increase or decrease in light production was observed in the Cytox strain, indicating clear genotoxicity and absence of cytotoxicity as is expected for the positive control substances.

3.3.1. In vitro genotoxicity screening of registered substances

Two out of the 22 registered substances with data available in the ECHA database, showed a positive result in the Vitotox® test, i.e. α-terpineol and 4-*tert*-octylphenol. In the absence of S9 metabolic fraction, α-terpineol induced a concentration dependent increase in the maximum S/N ratio recorded in the Genox strain (Figure 2A). The threshold value of 1.5 was however only exceeded at a concentration of 500 µM. When the bacteria of the Genox strain were exposed to concentrations of α-terpineol higher than 500 µM, the maximum S/N ratio recorded decreased due to cytotoxicity as reflected by the minimum S/N ratios lower than 0.8 recorded in the Cytox strain. In the presence of S9 metabolic fraction, α-terpineol induced a comparable effect with maximum S/N ratios exceeding the threshold value of 1.5 at concentrations of 1 and 2 mM. Similarly, the maximum S/N ratio decreased at higher concentrations due to the cytotoxic effect of α-terpineol. A concentration dependent increase was also observed in the maximum Genox/Cytox ratio's with values exceeding the threshold of 1.5, both in absence and in presence of S9 metabolic fraction (Figure 2B).

The compound 4-*tert*-octylphenol also induced a concentration dependent increase in the maximum S/N ratios recorded in the Genox strain but only in presence and not in absence of S9 metabolic fraction. Although an increase in the maximum S/N ratios is observed after exposure to 200 µM, 500 µM and 1 mM, the threshold value of 1.5 is only exceeded for the concentration of 2 mM (Figure 2C). For the Genox/Cytox ratios, a concentration dependent

increase is observed, both in the presence and the absence of S9 metabolic fraction. Although the maximum Genox/Cytox ratios observed in absence of S9 metabolic fraction clearly exceeded the threshold value of 1.5, interpretation of the potential genotoxic effect of 4-*tert*-octylphenol is complicated by the cytotoxic effect of the compound as observed in the Cytox strain. In presence of S9 metabolic fraction, genotoxicity of 4-*tert*-octylphenol was more pronounced despite the fact that the increase in maximum Genox/Cytox ratio's did not exceed the threshold value of 1.5 (Figure 2D).

3.3.2. In vitro genotoxicity screening of unregistered substances

None of the 26 substances with no data available in the ECHA database fulfilled the criteria for a positive result in the Vitotox® test.

3.4. Prioritization of substances based on the need for more data

Based on the information obtained from the ECHA database, the *in silico* predictions with ToxTree and Derek Nexus™ and the *in vitro* results of the Vitotox® test, the substances were classified into one of the three main groups according to the decision tree presented in Figure 1. As no independent evaluation reports are currently available, the absence of genotoxicity could not be unequivocally confirmed for any of the substances. Consequently, no substances were classified in group 3. On the other hand, no substance was assigned to group 1 as for none of the substances *in vivo* data were available on the ECHA website demonstrating genotoxicity. Thus, all 48 substances were classified in one of the four subgroups of group 2. Most of the substances showed a low (15) or medium (20) priority with regard to the need for additional genotoxicological information. For 12 of the substances, the necessity for more genotoxicity data is high, and for one substance, i.e. 2,4,6-trimethylbenzaldehyde, it is even very high (Figure 3).

4. Discussion

Since complete characterization of the genotoxic profile of the 48 substances that have been shown to migrate from PC-replacement baby bottles is impossible on short term, a strategy was developed in order to assign priority to those substances for which more data are urgently needed.

Although none of the 48 substances could be unequivocally identified as genotoxic (group 1), the presence of genotoxic effects could neither be excluded for any of them (group 3). Consequently, all 48 substances were classified in group 2, thus requiring more data to investigate the genotoxic potential. The relative need for such data, however, differed significantly and therefore, the 48 substances were further divided into four subgroups.

Substances classified in group 2D

For the substances of group 2D, the need for additional genotoxicity data is low. These are the substances for which the information from the ECHA database clearly confirms the absence of genotoxic effects and for which neither the *in silico* tools or the Vitotox® test provided indications for potential genotoxicity. However, since for none of these substances, an independent evaluation has been carried out by an EU member state, the presence of genotoxicity cannot be completely excluded. These substances require thus an independent assessment of the available data, rather than the generation of new genotoxicity data.

Substances classified in group 2C

Most of the 48 substances were classified in group 2C, showing a medium priority for more genotoxicity data. This group contains (i) substances for which no or insufficient data are available in the ECHA database and that do not trigger alerts for genotoxicity in the *in silico* tools or the Vitotox® test and (ii) substances for which data from the ECHA database support the absence of genotoxicity, but for which a positive result was obtained either with the *in silico* tools or with the Vitotox® test. The type of information requested may differ within this

group. Indeed, for substances that have not yet been registered under REACH, genotoxicity results are needed. In first instance, data on chromosome damage should be collected since the Vitotox® test was negative for these substances. Although the latter only provides information on DNA damage, results of the Vitotox® test strongly correlate with the results of the Ames test (± 90%) (Westerink et al., 2009) and thus provide indirect information on the induction of gene mutations. In contrast, the missing information should be collected for substances with insufficient data in the ECHA database.

For cyclohexanone, *in vitro* and *in vivo* data on chromosome damage are lacking or insufficient and therefore, an additional literature search using tools other than the ECHA database was performed. On Toxnet, *in vitro* micronucleus data were found indicating that cyclohexanone did not induce chromosome damage in bovine lymphocytes in the absence of S9 metabolic fraction (Piesova et al., 2003). Furthermore, cyclohexanone was used as a negative control for both *in vitro* and *in vivo* genotoxicity in the validation of the comet assay in the reconstructed 3D human epidermal skin models (Reus et al., 2013). All these data suggest that cyclohexanone is not genotoxic. This example illustrates that a targeted search for additional literature data on genotoxicity can help in setting priority for chemicals of concern.

For substances with sufficient information in the ECHA database supporting the absence of genotoxic effects and without SA for genotoxicity in the *in silico* tools, but which tested positively in the Vitotox® test, the positive result obtained in the Vitotox® test should be investigated further. One explanation may be that the DNA damage detected in the Vitotox® test is correctly repaired before the DNA damage is translated into stable mutations. This might for example be the case for 4-tert-octylphenol. The latter fulfilled the criteria for a positive response in the Vitotox® test, but only in presence of the S9 metabolic fraction. Induction of repairable DNA damage has been suggested for 4-octylphenol, a compound that is structurally related to 4-tert-octylphenol (Tayama et al., 2008). However, more data are needed to investigate whether the hypothesis of repairable DNA damage can explain the

discordance between our Vitotox® result and the previously reported Ames test results. Also for α-terpineol, another test compound that yielded a positive result in the Vitotox® test both in the presence and the absence of S9 metabolic fraction, more data were collected. Although all genotoxicity data available in the ECHA database were negative, positive results for α-terpineol in the Ames test have previously been reported (BIBRA Working Group, 2001; Gomes-Carneiro et al., 1998). It is also important to note that the purity of α-terpineol was only 90%. Genotoxicity observed in the Vitotox® test may thus be due to the presence of a genotoxic impurity.

Finally, substances that did not reveal any genotoxicity according to the ECHA data and showed a negative result in the Vitotox® test, but which triggered at least one SA for genotoxicity in ToxTree or Derek Nexus™ were also included in group 2C. Two substances fulfilled these criteria, among which isophorone was considered positive in both ToxTree and Derek Nexus™, whereas naphthalene was only identified as genotoxic in Derek Nexus™. Isophorone, as well as naphthalene, triggered a SA for the endpoint '*in vitro* chromosome damage' in Derek Nexus™. For both substances, this was in agreement with the positive results for *in vitro* chromosome damage reported in the ECHA database. However, for the two substances, the positive *in vitro* data were overruled by the negative result observed in the *in vivo* follow-up test. Thus, more data for these substances are not really requested although an independent evaluation should be performed to confirm the absence of genotoxic effects. For naphthalene, a (re-)evaluation of the information submitted by the industry in the context of REACH has been assigned to the UK and will start in 2016. This (re-)evaluation has been triggered by the contradictory information with respect to the carcinogenic effects of the substance. Naphthalene has been established as carcinogenic to both mice and rats in life-time inhalation exposure studies by the National Toxicology Program (NTP) (Abdo et al., 2001; NTP, 2000). However, since the information available for humans is limited, the International Agency for Research on Cancer has classified naphthalene as a possible human carcinogen (IARC, 2002). Furthermore, at present it remains unclear whether a genotoxic mode of action

is involved in the possible carcinogenic effects of naphthalene although most evidence indicates that naphthalene is not genotoxic (Brusick, 2008).

Substances classified in group 2B

Substances of group 2B were assigned a high priority for more information on genotoxicity. These are (i) substances for which no data are available in the ECHA database and for which a positive result was obtained either with the *in silico* tools or with the Vitotox® test and (ii) substances with data in the ECHA database supporting the lack of genotoxicity, but triggering positive results both with the *in silico* tools and in the Vitotox® test. All but one of the substances classified in group 2B were non-registered substances that triggered at least one SA in ToxTree and/or Derek Nexus™. For these substances, data on chromosome damage should first be collected since they were all negative in the Vitotox® test. Furthermore, the both substances that were identified as potentially genotoxic in the two *in silico* tools, i.e. 3,5-di-tert-butylbenzoquinone and 2,6-di(tert-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one, triggered and SA in Derek Nexus™ for the endpoint '*in vitro* chromosome damage' and '*in vitro* and *in vivo* chromosome damage', respectively. Interestingly, all seven substances that triggered a SA for 'genotoxic carcinogenicity' in ToxTree but not for any of the genotoxicity-related endpoints in Derek Nexus™, were benzaldehydes. The aldehyde-function is indeed a SA for genotoxic carcinogenicity of the Benigni-Bossa module (Benigni et al., 2008). Experimental evidence has demonstrated the genotoxic and carcinogenic activity of some simpler aldehydes e.g. formaldehyde, acetaldehyde and furfural (Feron et al., 1991). Benzaldehyde, on the other hand, can be found in natural fruit flavours and is generally recognized as safe (GRAS) as a food additive in both the US and Europe (Andersen, 2006). This substance, like other types of benzaldehydes, was negative in the Ames test, but produced chromosome damage *in vitro*. The *in vivo* studies with benzaldehyde (and other types of benzaldehydes) for chromosome damage were however all negative (EFSA, 2010). It has been suggested that aldehydes, including benzaldehydes, exhibit a short plasma half-life

and are efficiently oxidized to the corresponding acids, which are metabolized in the fatty acid or citric acid pathways. These *in vivo* conditions cannot be mimicked *in vitro*, and this may consequently explain the different response observed *in vitro* and *in vivo* (JECFA/WHO, 1998). Benzaldehyde is thus generally accepted as non-genotoxic and this is probably the reason why the benzaldehydes did not trigger a SA in Derek Nexus^{TM}. Nevertheless, for many benzaldehydes, there are only limited data, so more research is needed to investigate whether other benzaldehydes may also be considered non-genotoxic. On the other hand, the three substances that were predicted to induce chromosome damage by Derek Nexus^{TM} but that were not identified as 'genotoxic carcinogens' by ToxTree were all naphthalene derivatives. As discussed above, naphthalene triggered chromosome damage *in vitro* which was not confirmed by most of the *in vivo* studies. However, as none of the three substances has been registered under REACH, the genotoxicity of the naphthalene derivatives should be investigated in more detail. The other substance classified in group 2B was 2-ethylhexyl acrylate. For this substance, the *in vitro* genotoxicity data reported in the ECHA database were ambiguous and not supplemented by adequate *in vivo* studies. Furthermore, the substance triggered a SA in Derek Nexus^{TM} for *in vitro* chromosome damage. Consequently, more *in vitro* and *in vivo* data on chromosome damage should be collected for this substance.

Substances classified in group 2A

Finally, there was one substance classified in group 2A, i.e. 2,4,6-trimethylbenzaldehyde. In analogy with the other benzaldehydes, the substance triggered a SA for genotoxic carcinogenicity in ToxTree and the *in vitro* genotoxicity data from the ECHA database indicated that the substance was negative in the Ames test but produced chromosome aberrations. However, *in vivo* genotoxicity data are lacking. Consequently, it needs to be further investigated whether this benzaldehyde is indeed not genotoxic *in vivo*.

Strengths and limitations

Despite the usefulness of the strategy, some limitations should however be kept in mind. First, for none of the substances included in the present study, the data available in the ECHA database have been evaluated independently. Second, negative predictions for genotoxic carcinogenicity with ToxTree and for chromosome damage with Derek Nexus™ should be interpreted with caution. Negative predictions do not necessarily mean that these compounds are not genotoxic; they may be non-genotoxic, but they may also be outside the model's domain. Third, the Vitotox® test is an indicator test and thus does not detect permanent DNA damage. Interestingly, none of the 48 substances triggered a SA for gene mutations in Derek Nexus™. The lack of these SA correlates well with the results of the Vitotox® test. Indeed, only two compounds showed a positive result in the Vitotox® test, and as discussed above, there may be an explanation why these substances are positive in the Vitotox® test and not in the Ames test. Finally, there is one substance that may appear to be classified incorrectly, i.e. dicyclopentyl(dimethoxy)silane. Indeed, this substance is assigned to group 2D, although the *in vivo* genotoxicity data available in the ECHA database are equivocal. In the proposed decision tree, *in vivo* genotoxicity data are not considered in case not-equivocal and sufficient *in vitro* data demonstrate that the substance is not genotoxic. This is based on the hypothesis that almost all *in vivo* genotoxicants are also detected in (a battery of) *in vitro* tests. For dicyclopentyl(dimethoxy)silane, the *in vivo* data for chromosome damage are obtained from read-across with the results of an *in vivo* micronucleus test with cyclohexyl-dimethoxymethylsilane. Results of read-across should always be interpreted with caution. Furthermore, this substance only induced an increase in micronuclei in the polychromatic erythrocytes of male mice at the highest dose, a dose that also induced toxicity. No genotoxic effect was observed in females. Together with the lack of any SAs in the *in silico* tools and a negative result in the Vitotox® test, dicyclopentyl(dimethoxy)silane can indeed be considered as a substance of low genotoxic concern.

5. Conclusions

The results of the current study show that by applying a strategy based on information available in the ECHA database, *in silico* prediction tools and a screening test for DNA damage, different priority levels can be assigned to substances migrating from PC-replacement baby bottles. Interestingly, the application of this strategy is not limited to PC-replacement baby bottles, even not to the domain of food contact materials, but can be broadened to any group of chemicals. Although the strategy is not suited, nor intended, to replace the data required in a regulatory context, the current study illustrates that it can be of particular interest to identify chemicals of concern within a large number of compounds.

6. Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Figure captions

Figure 1. Decision tree for the grouping of substances using the genotoxicity data collected from the ECHA database, the *in silico* predictions with ToxTree and Derek Nexus™ and the results of the Vitotox® test. Group 3 includes non-genotoxic substances for which no further genotoxicity data are required; Group 2 represents substances for which more genotoxicity data are needed; and Group 1 contains genotoxic substances for which migration from the baby bottles needs to be further investigated. Substances of group 2 can be further divided into four subgroups depending on the urgency of the need for further genotoxicity data with (i) subgroup 2A: very high priority for more genotoxicity data; (ii) subgroup 2B: high priority for more genotoxicity data; (iii) subgroup 2C: medium priority for more genotoxicity data and subgroup 2D: low priority for more genotoxicity data.

Figure 2. Results of the Vitotox®-test for α-terpineol and 4-tert-octylphenol. (A,C) Maximum and minimum S/N ratio recorded in 4 h in the Genox strain and the Cytox strain respectively and expressed as a function of the concentration of α-terpineol (A) and 4-tert-octylphenol (C). (B, D) Maximum Genox/Cytox ratio recorded in 4 h expressed as a function of the concentration of α-terpineol (B) and 4-tert-octylphenol (D). Experiments were performed in presence and absence of S9 metabolic fraction. 4-nitroquinoline (4-NQO) is the positive control in absence of S9 and benzo(a)pyrene (BaP) is the positive control in presence of S9.

Figure 3. Result of the grouping of the 48 substances according to the decision tree presented in Figure 1. All substances were divided into one of the four subgroups of group 2. For this reason, group 1 and group 3 are not included in the figure.

Table captions

Table 1. Overview of the 48 test compounds included in the current study. Test compounds were selected based on the data from Simoneau et al. (2012) and Onghena et al. (2014 and 2015).

Table 2. Description of the different categories and subcategories of mutagens according to the CLP Regulation (European Union 2008).

Table 3. Overview of the information collected from the ECHA database for the 22 registered test substances. NA: no information available; +: equivocal or insufficient; P: evaluation by a European member state is planned in the future but has not yet started; O: evaluation study is currently on-going.

Table 4. In silico predictions on the genotoxic potential of test compounds for which data are available in the ECHA database using ToxTree and Derek Nexus^{TM}.

Table 5. In silico predictions on the genotoxic potential of test compounds for which no data are available in the ECHA database using ToxTree and Derek Nexus^{TM}.

Figure 1

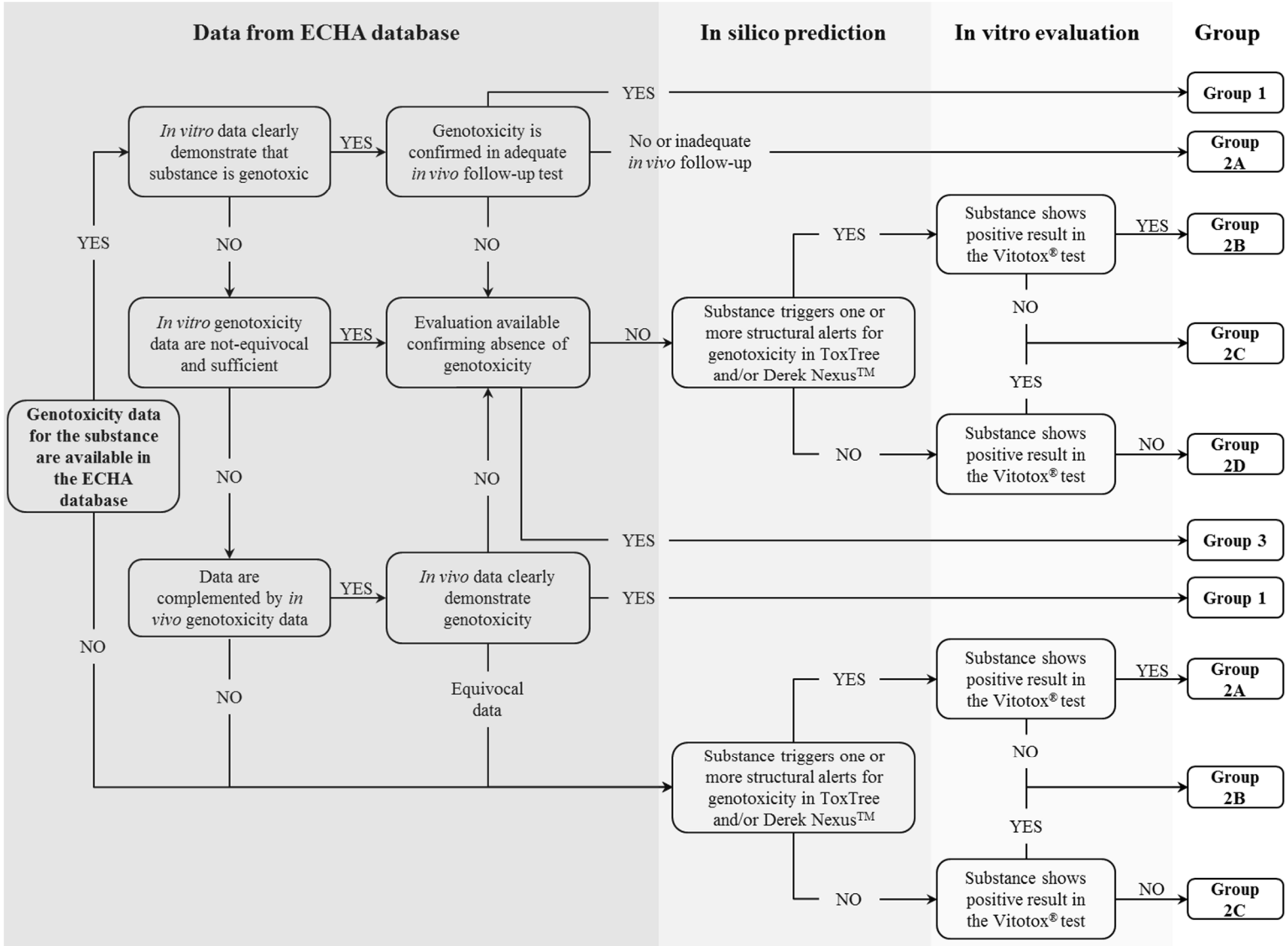
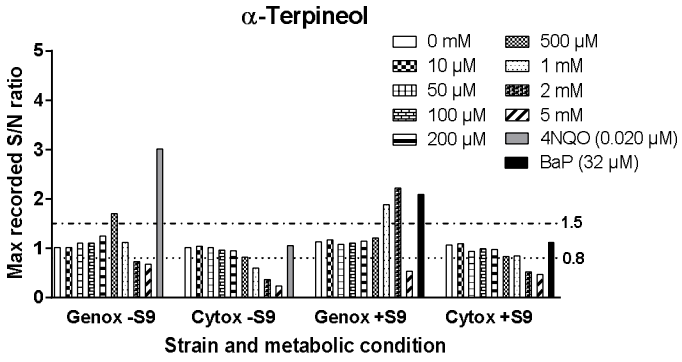
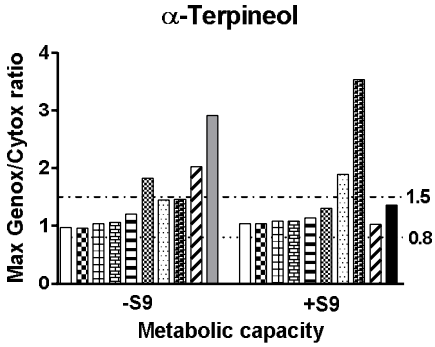

Data from ECHA database
In silico prediction
In vitro evaluation
Group
Genotoxicity data for the substance are available in the ECHA database
In vitro data clearly demonstrate that substance is genotoxic
YES
Genotoxicity is confirmed in adequate in vivo follow-up test
YES
No or inadequate in vivo follow-up
Group 1
Group 2A
NO
NO
In vitro genotoxicity data are not-equivocal and sufficient
YES
Evaluation available confirming absence of genotoxicity
NO
Substance triggers one or more structural alerts for genotoxicity in ToxTree and/or Derek Nexus™
YES
Substance shows positive result in the Vitotox® test
YES
Group 2B
NO
Group 2C
YES
NO
Substance shows positive result in the Vitotox® test
NO
Group 2D
NO
NO
YES
Group 3
Data are complemented by in vivo genotoxicity data
YES
In vivo data clearly demonstrate genotoxicity
YES
Group 1
NO
NO
Equivocal data
Substance triggers one or more structural alerts for genotoxicity in ToxTree and/or Derek Nexus™
YES
Substance shows positive result in the Vitotox® test
YES
Group 2A
NO
Group 2B
YES
NO
Substance shows positive result in the Vitotox® test
NO
Group 2C

Figure 2

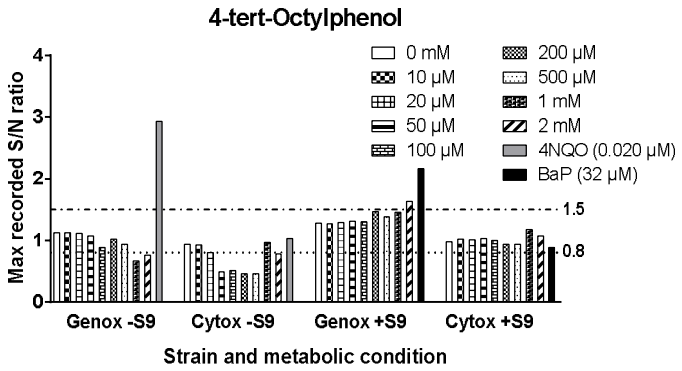
A.



B.



C.



D.

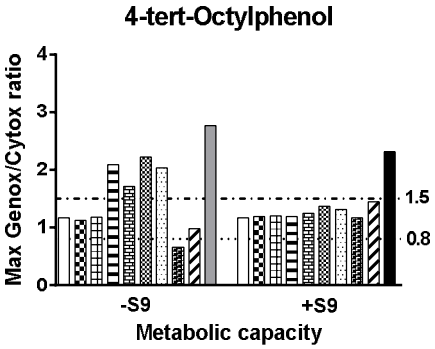


Figure 3

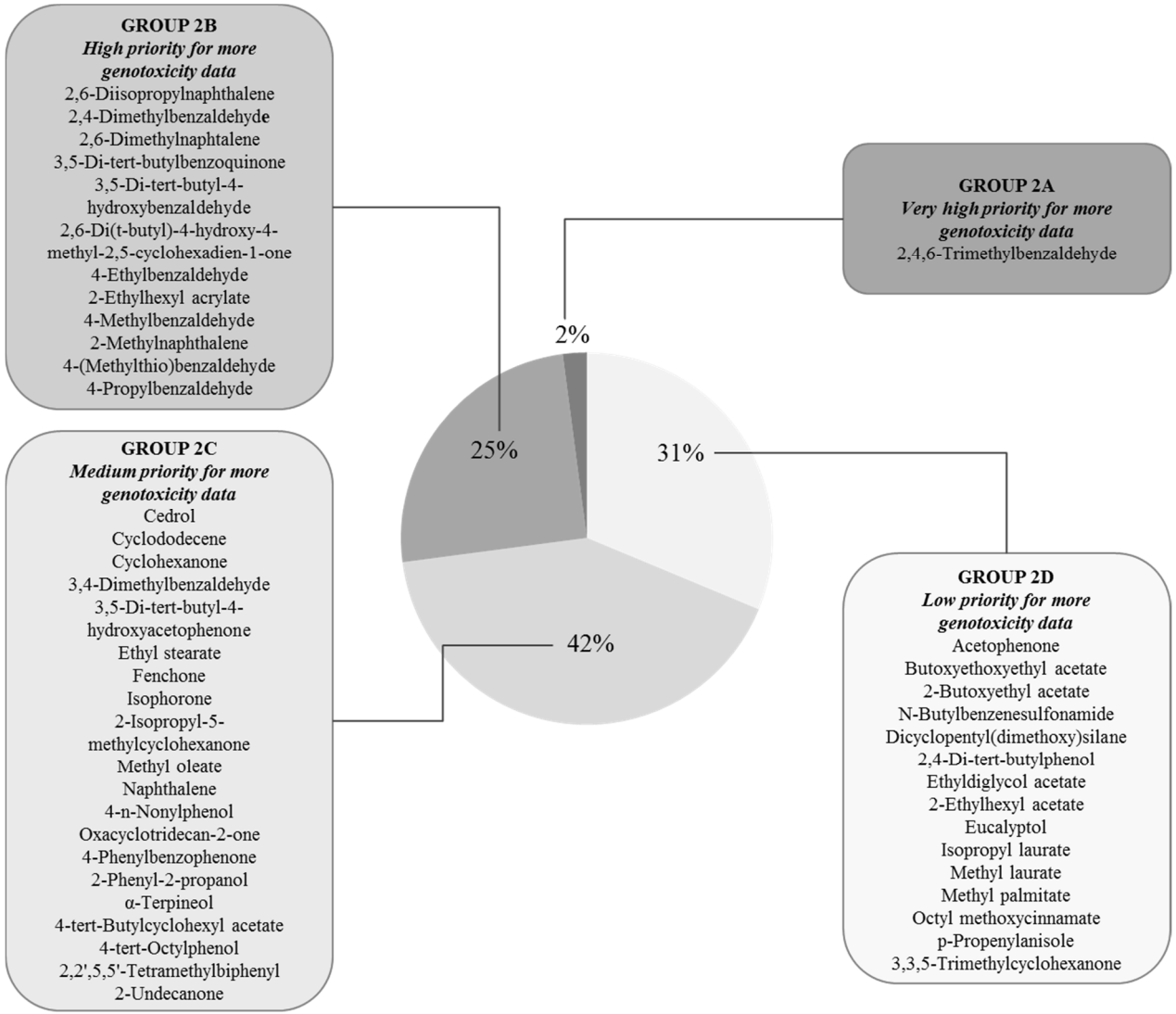

GROUP 2B
High priority for more genotoxicity data
2,6-Diisopropylnaphthalene
2,4-Dimethylbenzaldehyde
2,6-Dimethylnaphtalene
3,5-Di-tert-butylbenzoquinone
3,5-Di-tert-butyl-4-hydroxybenzaldehyde
2,6-Di(t-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one
4-Ethylbenzaldehyde
2-Ethylhexyl acrylate
4-Methylbenzaldehyde
2-Methylnaphthalene
4-(Methylthio)benzaldehyde
4-Propylbenzaldehyde
GROUP 2A
Very high priority for more genotoxicity data
2,4,6-Trimethylbenzaldehyde
2%
25%
31%
42%
GROUP 2C
Medium priority for more genotoxicity data
Cedrol
Cyclododecene
Cyclohexanone
3,4-Dimethylbenzaldehyde
3,5-Di-tert-butyl-4-hydroxyacetophenone
Ethyl stearate
Fenchone
Isophorone
2-Isopropyl-5-methylcyclohexanone
Methyl oleate
Naphthalene
4-n-Nonylphenol
Oxacyclotridecan-2-one
4-Phenylbenzophenone
2-Phenyl-2-propanol
α-Terpineol
4-tert-Butylcyclohexyl acetate
4-tert-Octylphenol
2,2',5,5'-Tetramethylbiphenyl
2-Undecanone
GROUP 2D
Low priority for more genotoxicity data
Acetophenone
Butoxyethoxyethyl acetate
2-Butoxyethyl acetate
N-Butylbenzenesulfonamide
Dicyclopentyl(dimethoxy)silane
2,4-Di-tert-butylphenol
Ethyldiglycol acetate
2-Ethylhexyl acetate
Eucalyptol
Isopropyl laurate
Methyl laurate
Methyl palmitate
Octyl methoxycinnamate
p-Propenylanisole
3,3,5-Trimethylcyclohexanone

Table 1. Overview of the 48 test compounds.

Chemical substances (Current name)	**CAS**	**Provider**	**Purity**
Acetophenone	98-86-2	Sigma	≥99%
Butoxyethoxyethyl acetate	124-17-4	Sigma	≥99.2%
2-Butoxyethyl acetate	112-07-2	Sigma	99%
N-Butylbenzenesulfonamide	3622-84-2	Sigma	99%
Cedrol	77-53-2	Sigma	Not specified by manufacturer
Cyclododecene	1501-82-2	Sigma	96%
Cyclohexanone	108-94-1	Sigma	≥99.5
Dicyclopentyl(dimethoxy)silane	126990-35-0	TCI	98%
2,6-Diisopropylnaphthalene (DIPN)	24157-81-1	Sigma	>99%
2,4-Dimethylbenzaldehyde	15764-16-6	Sigma	≥90%
3,4-Dimethylbenzaldehyde	5973-71-7	Sigma	98%
2,6-Dimethylnaphtalene	28804-88-8	Sigma	≥90%
3,5-Di-tert-butylbenzoquinone	719-22-2	Sigma	98%
3,5-Di-tert-butyl-4-hydroxyacetophenone	14035-33-7	Sigma	Not specified by the manufacturer
3,5-Di-tert-butyl-4-hydroxybenzaldehyde	1620-98-0	Chemos	Not specified by the manufacturer
2,6-Di(tert-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	10396-80-2	Chemos	98%
2,4-Di-tert-butylphenol	96-76-4	Sigma	99%
4-Ethylbenzaldehyde	4748-78-1	Sigma	≥97%
Ethyldiglycol acetate	112-15-2	Sigma	99%
2-Ethylhexyl acetate	103-09-3	Sigma	≥99%
2-Ethylhexyl acrylate	103-11-7	Sigma	98%
Ethyl stearate	111-61-5	Sigma	>97%
Eucalyptol	470-82-6	Sigma	99%
Fenchone	7787-20-4	Sigma	≥98%
Isophorone	78-59-1	Sigma	97%
Isopropyl laurate	10233-13-3	Sigma	Not specified by the manufacturer
2-Isopropyl-5-methylcyclohexanone	10458-14-7	Sigma	≥97%
4-Methylbenzaldehyde	104-87-0	Sigma	≥97%
Methyl laurate	111-82-0	Sigma	99.5%
2-Methylnaphthalene	91-57-6	Sigma	97%
Methyl oleate	112-62-9	Sigma	≥99%
Methyl palmitate	112-39-0	Sigma	>97%
4-(Methylthio)benzaldehyde	3446-89-7	Sigma	95%
Napthalene	91-20-3	Sigma	99%
4-n-Nonylphenol	104-40-5	Sigma	≥96%
Octyl methoxycinnamate	5466-77-3	Sigma	98%
Oxacyclotridecan-2-one	947-05-7	Sigma	98%
4-Phenylbenzophenone	2128-93-0	Sigma	99%
2-Phenyl-2-propanol	617-94-7	Sigma	97%
4-Propylbenzaldehyde	28785-06-0	Sigma	97%
p-Propenylanisole	4180-23-8	Sigma	≥99.5%
α-Terpineol	98-55-5	Sigma	≥90%
4-tert-Butylcyclohexyl acetate	32210-23-4	Sigma	99%
4-tert-Octylphenol	140-66-9	Sigma	Not specified by manufacturer
2,2',5,5'-Tetramethylbiphenyl	3075-84-1	Chemos	98%
2,4,6-Trimethylbenzaldehyde	487-68-3	Sigma	98%
3,3,5-Trimethylcyclohexanone	873-94-9	Sigma	98%
2-Undecanone	112-12-9	Sigma	99%

Table 2. Description of the different categories and subcategories of mutagens according to the CLP Regulation (European Union 2008).

	Category	Description
Mutagens	Cat 1A	Substances known to induce heritable mutations in the germ cells of humans.
	Cat 1B	Substances to be regarded as if they induce heritable mutations in the germ cells of humans.
	Cat 2	Substances which cause concern for humans owing to the possibility that they may induce heritable mutations in the germ cells of humans

Table 3. Overview of the information collected from the ECHA database for the 22 registered test substances. NA: no information available; ±: equivocal or insufficient; P: evaluation by a European member state is planned in the future but has not yet started; O: evaluation study is currently on-going.

Chemical name	*In vitro* genotoxicity			*In vivo* genotoxicity			Final call	Evaluation	Harmonized CLP Classification for Mutagenicity
	Gene mutations	Chromosome damage	Non-specific genotoxicity	Gene mutations	Chromosome damage	Non-specific genotoxicity			
Acetophenone	-	+	-	NA	-	NA	-	NA	NA
Butoxyethoxyethyl acetate	-	-	NA	NA	NA	NA	-	NA	NA
2-Butoxyethyl acetate	-	-	NA	NA	-	NA	-	NA	NA
N-Butylbenzenesulfonamide	-	-	NA	NA	NA	NA	-	NA	NA
Cyclohexanone	-	NA	-	NA	±	NA	±	NA	NA
Dicyclopentyl(dimethoxy)silane	-	-	NA	NA	±	NA	±	NA	NA
2,4-Di-tert-butylphenol	-	±	NA	NA	-	NA	-	P	NA
Ethyldiglycol acetate	-	NA	NA	NA	-	NA	-	NA	NA
2-Ethylhexyl acetate	-	NA	-	NA	-	NA	-	O	NA
2-Ethylhexyl acrylate	±	±	±	NA	NA	±	±	NA	NA
Eucalyptol	-	-	±	NA	-	NA	-	NA	NA
Isophorone	±	±	±	NA	-	NA	±	O	NA
Isopropyl laurate	-	-	NA	NA	NA	NA	-	NA	NA
Methyl laurate	-	-	NA	NA	NA	NA	-	NA	NA
Methyl palmitate	-	-	NA	NA	NA	NA	-	NA	NA
Naphthalene	-	+	+	NA	-	-	-	P	NA
Octyl methoxycinnamate	-	-	-	NA	-	NA	-	P	NA
p-Propenylanisole	-	-	-	NA	NA	-	-	NA	NA
α-Terpineol	-	-	NA	NA	NA	NA	-	NA	NA
4-tert-Octylphenol	-	-	NA	NA	NA	NA	-	NA	NA
2,4,6-Trimethylbenzaldehyde	-	+	NA	NA	NA	NA	±	NA	NA
3,3,5-Trimethylcyclohexanone	-	+	NA	NA	-	NA	-	NA	NA

Table 4. *In silico* predictions on the genotoxic potential of test compounds for which data are available in the ECHA database using ToxTree and Derek Nexus™.

Chemical name	ToxTree	Derek Nexus™			Min 1 SA for genotoxicity
	Genotoxic carcinogenicity	Gene mutations	Chromosome damage	Non-specific genotoxicity	
Acetophenone	-	-	-	-	-
2-Butoxyethyl acetate	-	-	-	-	-
Butoxyethoxyethyl acetate	-	-	-	-	-
N-Butylbenzenesulfonamide	-	-	-	-	-
Cyclohexanone	-	-	-	-	-
Dicyclopentyl(dimethoxy)silane	-	-	-	-	-
2,4-Di-tert-butylphenol	-	-	-	-	-
Ethyldiglycol acetate	-	-	-	-	-
2-Ethylhexyl acetate	-	-	-	-	-
2-Ethylhexyl acrylate	-	-	+	-	+
Eucalyptol	-	-	-	-	-
Isophorone	+	-	+	-	+
Isopropyl laurate	-	-	-	-	-
Methyl laurate	-	-	-	-	-
Methyl palmitate	-	-	-	-	-
Naphthalene	-	-	+	-	+
Octyl methoxycinnamate	-	-	-	-	-
p-Propenylanisole	-	-	-	-	-
3,3,5-Trimethylcyclohexanone	-	-	-	-	-
2,4,6-Trimethylbenzaldehyde	+	-	-	-	+
α-Terpineol	-	-	-	-	-
4-tert-Octylphenol	-	-	-	-	-

Table 5. *In silico* predictions on the genotoxic potential of test compounds for which no data are available in the ECHA database using ToxTree and Derek Nexus™

Chemical name	ToxTree	Derek Nexus™			Min 1 SA for genotoxicity
	Genotoxic carcinogenicity	Gene mutations	Chromosome damage	Non-specific genotoxicity	
Cedrol	-	-	-	-	-
Cyclododecene	-	-	-	-	-
2,6-Diisopropylnaphthalene	-	-	+	-	+
2,4-Dimethylbenzaldehyde	+	-	-	-	+
3,4-Dimethylbenzaldehyde	+	-	-	-	+
2,6-Dimethylnaphtalene	-	-	+	-	+
3,5-Di-tert-butylbenzoquinone	+	-	+	-	+
3,5-Di-tert-butyl-4-hydroxyacetophenone	-	-	-	-	-
3,5-Di-tert-butyl-4-hydroxybenzaldehyde	+	-	-	-	+
2,6-Di(tert-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	+	-	+	-	+
4-Ethylbenzaldehyde	+	-	-	-	+
Ethyl stearate	-	-	-	-	-
Fenchone	-	-	-	-	-
2-Isopropyl-5-methylcyclohexanone	-	-	-	-	-
4-Methylbenzaldehyde	+	-	-	-	+
2-Methylnaphthalene	-	-	+	-	+
Methyl oleate	-	-	-	-	-
4-(Methylthio)benzaldehyde	+	-	-	-	+
4-n-Nonylphenol	-	-	-	-	-
Oxacyclotridecan-2-one	-	-	-	-	-
4-Phenylbenzophenone	-	-	-	-	-
2-Phenyl-2-propanol	-	-	-	-	-
4-Propylbenzaldehyde	+	-	-	-	+
4-tert-Butylcyclohexyl acetate	-	-	-	-	-
2,2',5,5'-Tetramethylbiphenyl	-	-	-	-	-
2-Undecanone	-	-	-	-	-