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Exploring BPA Alternatives – Environmental Levels and Toxicity Review

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Abstract:

Bisphenol A alternatives are manufactured as potentially less harmful substitutes of bisphenol A (BPA) that offer similar functionality. These alternatives are already in the market, entering the environment and thus raising ecological concerns. However, it can be expected that levels of BPA alternatives will dominate in the future, they are limited information on their environmental safety. The EU PARC project highlights BPA alternatives as priority chemicals and consolidates information on BPA alternatives, with a focus on environmental relevance and on the identification of the research gaps. The review highlighted aspects and future perspectives. In brief, an extension of environmental monitoring is crucial, extending it to cover BPA alternatives to track their levels and facilitate the timely implementation of mitigation measures. The biological activity has been studied for BPA alternatives, but in a non-systematic way and prioritized a limited number of chemicals. For several BPA alternatives, the data has already provided substantial evidence regarding their potential harm to the environment. We stress the importance of conducting more comprehensive assessments that go beyond the traditional reproductive studies and focus on overlooked relevant endpoints. Future research should also consider mixture effects, realistic environmental concentrations, and the long-term consequences on biota and ecosystems.

Keywords: BPA alternatives, biological activity, *in silico*, invertebrates, vertebrates

Table of context

1. Introduction

Bisphenol A (BPA) has garnered significant attention due to its endocrine disruption activity and

adverse health effects on humans [1] and other organisms even in environmentally realistic concentrations [2,3]. However, so far due to the lack of appropriate studies and knowledge there is no evidence of effects at ecosystem level. Research has shown that BPA can act as an endocrine disruptor, interfering with hormone signaling pathways in the body, which was associated with a range of health concerns, including reproductive disorders, developmental abnormalities and metabolic changes among others [4]. While regulatory measures have been put in place to limit the exposure to BPA, the potential risks associated with its toxicity continue to be an area of ongoing research and concern for human and environmental health. In response to proposed and existing regulatory restrictions, manufacturers of BPA-containing products sought out potentially less harmful substitutes that offer similar functionality. These chemicals are commonly known as BPA alternatives. These alternatives were perceived as safer options and believed to have a more favorable toxicity profile. However, later studies have shown that many BPA alternatives may possess similar endocrine-disruptive or other adverse properties, suggesting that there is a lack of information for assessing their safety [5]. Despite existing and ongoing research efforts on BPA alternatives, there is a significant research need to better understand the toxicity of these alternatives and to identify and develop safer materials for use in consumer products. The goal is to ensure that the replacements for BPA do not compromise human health or the environment while maintaining the necessary functionalities that consumers rely on.

Many of these BPA alternative substances are already on the market and are frequently found in the environment, although the information about their bioactivity is scattered and likely incomplete. Human activities, such as industrial processes, product manufacturing, and improper disposal, contribute to the release of BPA alternatives into the environment. Once released, these chemicals can enter the ecosystem through various pathways, such as industrial discharges, wastewater treatment plants, and leaching from landfills. The available monitoring studies show the presence of BPA alternatives in the environment and raise concerns about their potential ecological impacts. European Agencies [e.g. European Chemicals Agency (ECHA), European Food Safety Authority (EFSA)] recognized the increasing concerns about BPA and its alternatives, therefore scientists have prioritized research on BPA alternatives, and recently, the PARC project (Partnership for the Assessment of Risks from Chemicals) has also recognized the importance of this issue [6]. In line with PARC's objectives, our aim is to consolidate existing information on the scope of the BPA alternatives problem by gaining a comprehensive understanding of their detectable levels in the environment. Furthermore, our review outlines existing methodologies and approaches employed to assess the bioactivity of BPA alternatives which use existing databases and *in silico* tools as well as invertebrate and vertebrate models that are relevant to the natural environment. By doing so, we aim to identify any research gaps that may be of significance to environmental toxicologists, industry scientists, and regulators. Additionally, gaining mechanistic insights into the disruptive effects of BPA alternatives will aid in selecting chemicals that are suitable for the development of new toxicity assessment methods, which is one of the key priorities for PARC. Finally, the identification of knowledge gaps on the potential toxicity of BPA alternatives in different organisms' categories will reveal the need to focus on additional endpoints other than the ones already investigated.

2. Methodology

2.1. Studies on environmental levels

In the present review we performed a literature search for the identification of the most relevant studies with monitoring data of bisphenol's mixtures in different environmental compartments. The literature search was carried out in 2023 and followed the general principles of the European Food Safety Authority (EFSA) Guidance for systematic literature review (EFSA, 2010). The main objective of

this literature review was the collection of monitoring data for bisphenols in different environmental compartments, i.e., water (surface fresh water and sea water), soil and sediment, across Europe.

Considering the overall objectives of the literature review, specific inclusion and exclusion criteria were set and followed for the screening of the identified references and the identification of the most relevant studies to be reviewed in detail (Table 1).

Language	IN	English
Type of studies	IN	Monitoring studies in different environmental compartments
	OUT	Studies regarding method validation (unless real samples are analyzed)
Location	IN	European countries
	OUT	USA, Asian and other non-EU countries
Type of monitoring data	IN	Monitoring data for BPA alternatives
	OUT	Monitoring data only for BPA
Analyzed matrices	IN	Water, surface water, groundwater, seawater, freshwater, sediment, soil
	OUT	Air, wastewater, biological fluids (samples from biomonitoring studies), sewage sludge, sewage treatment plants, urban run-off, drinking water stored in bottles

Tab.1: Inclusion / exclusion criteria for literature search for the collection of monitoring data of bisphenols.

2.2. *In silico* approaches and toxicity studies

2.2.1. Chemical identifiers

The full names, long and short abbreviations of BPA and 25 BPA alternatives together with their chemical identifiers (DTXSID, CASRN) and molecular weights are given in Table 1.

2.2.2. Physicochemical properties

The octanol-water partition coefficient of the neutral species (logK_{ow}) were collected from literature.

Wherever possible, experimental values were preferred, however, if not available, estimates were recorded from the four prediction models in the Chemistry Dashboard (US EPA, 2023) and the predictions from KOWWIN v1.67 from Chemspider (2023) and an average of the predicted logK_{ow} were used for the prediction of the liposome-water partition constants.

The acidity constants pK_a for bisphenol S (BPS) and tetrabromobisphenol A (TBBPA) were measured using a Sirius T3 titrator (Niu et al. 2022) and the remaining compounds were predicted with ACD/Percepta (2015), using the GALAS algorithm The fraction of neutral species was calculated with eq. 1 for monoprotic acids and according to Escher et al. 2020 for diprotic acids [7].

$$\alpha_{neutral} = \frac{1}{1+10^{pH-pK_a}} \quad (1)$$

The logK_{ow} of all neutral species was converted to the liposome-water partition constant log K_{lipw} with the Quantitative Structure-Activity Relationship (QSAR) established by Endo et al. (eq. 2).

$$\log K_{lipw} = 1.01 \times \log K_{ow} + 0.12 \quad \text{for } -1 < \log K_{ow} < 8 \quad (2)$$

The K_{lipw} relates to the neutral species but some of the BPA alternatives are partially ionized. The D_{lipw}(pH 7.4) is considered a better predictor of uptake into cells and organisms than the ionization-corrected K_{ow} (Escher et al. 2020a). The D_{lipw} (pH 7.4) was estimated with an equation from Escher et al. (2020a).

$$D_{lipw}(\text{pH 7.4}) = K_{lipw}(\text{neutral species}) \times [\alpha_{neutral} + 0.1 \times (1 - \alpha_{neutral})] \quad (3)$$

2.2.3 *In vitro* bioassay data

All concentration-response data for *in vitro* cell-based bioassays were downloaded from the cHTS database included in CurveSurfer which is part of the Integrated Chemical Environment (ICE) on the National Toxicology Program (NTP) of the U.S. Department of Health and Human Services (Abendini et al. 2021). Only cell-based assays from Tox21 and Attagene (ATG) assays were included in the analysis. The code for the R-script used to filter the Tox21 data, fit the corresponding concentration-response curves and calculate EC_{10} values is described in detail by Braun and Escher (2023) and is published on GitHub (https://github.com/braungeorg/NeuroMixPrioritization.git). R version 4.1.3 has to be used in combination with RStudio version 2021.9.2.382. The following improvements have been added to the R-script: Instead of using the relative AC_{50} value as threshold for testing for acceptable data point distributions of the concentration response curves (CRC), the EC_{10}, where 10% stands for 10% of the maximum effect, was used. For the Tox21 assays the maximum was 1 (100%). The assays from Attagene did not have a typical response maximum of 100% but were recorded as a log2-fold response induction, which had to be converted first to % by deriving the experimental maximum of 3.5 of all Attagene assays from the 95% quantile of all maxima. Therefore, the maximum was set to 3.5 for all ATG assays and the EC_{10} refers to a log2-fold response induction value of 0.35.

2.2.4 Baseline toxicity prediction

Baseline toxicity is the minimal toxicity that any chemical exhibits. For human cell lines the IC_{10} baseline can be predicted from the D_{lipw} (pH 7.4) with the QSAR given by eq. 4 established in our lab (Lee et al. 2021). Most baseline toxicity QSARs are linear with hydrophobicity but the baseline toxicity QSAR for cell lines is based on nominal concentrations and the bend in the QSAR is caused by binding of hydrophobic chemicals to medium components.

$$IC_{10,\,baseline}(M)=\frac{1}{10^{1.23+4.97\times\left(1-e^{-0.236\times D_{lipw}}\right)}} \quad (4)$$

2.2.5. Evaluation of specificity of effects

By comparing the prediction for baseline toxicity, expressed as inhibitory concentration IC_{10} for 10% cytotoxicity, with experimental EC_{10} one can identify which effects are caused by baseline toxicity and which effects are specific. The reporter gene will then give information about what type of specific effect is relevant. The specificity ratio $SR_{baseline}$ is a measure for how much more potent than baseline toxicity and therefore how specific a chemical is. It can be calculated with eq. 5 (Escher et al. 2020b).

$$Specificity\,Ratio\,SR_{baseline}=\frac{IC_{10,baseline}}{EC_{10}} \quad (5)$$

For those assays that also have cytotoxicity measured, the toxic ratio TR (Maeder et al. 2004) as the ratio between the baseline toxicity prediction IC_{10}, baseline and the experimental cytotoxicity IC_{10} (eq. 6) can be defined.

$$Toxic\,Ratio\,TR=\frac{IC_{10,baseline}}{IC_{10}} \quad (6)$$

2.3. Microbial organisms as single species or microbiomes

The literature review was performed on Web of Science by using “all fields” research with the following word sequence: (“BPA” OR “BPA alternatives” OR “BPA substitutes”) AND (“microbes” OR “microbiomes” OR “microbial organism” OR “microbial communities”). From this search, additional cited articles (not found in WoS) were examined for their relevance to the topic.

2.4. Studies investigating invertebrate models

2.4.1 Terrestrial and aquatic insects studies

The literature search for toxicity data of bisphenols and its alternatives to insects was performed by combining the search terms “insects” or “terrestrial insects” and “bisphenols” or “BPA alternatives”. The search was carried out in Scopus database.

2.4.2 Gastropods (Molluscs) studies

The following alternatives have been included in the literature search (mollus* AND bisphenol/ isopropylidenediphenol/ pergafast/ BADGE/ BPS/ BPF/ BPE/ BPAP/ BPAF/ BPZ/ TBBPA).

2.4.3 Ascidian studies

The literature search for toxicity data in Ciona and ascidians of bisphenols and its alternatives

was performed in the Web of Science (WoS) database by applying sequences of search terms separated by with Boolean operators (i.e., AND, OR, NOT or AND NOT). Searches were done in a broad manner by engaging the "Topic" domains in the "All Databases" option and the "All fields" domains in the "WoS Core Collection" option. Search terms identifying ascidians were: "Ciona" OR "Phallusia" OR "ascidian*" OR "tunicate*"). As search terms for chemicals the chemical identifiers listed in Table 2 were used (popular names, IUPAC-names, abbreviations, short abbreviations, and the CAS Registry Numbers). Star-mark (*) was used to avoid singular/plural issues. All articles that were returned based on the above search criterions were filtered by relevance-assessing the title, abstract and whole paper (when accessible). Articles that were found to be not relevant were removed. Articles remaining after filtering (both regular articles and reviews) were examined and data/information were harvested and further processed/analyzed for this study.

2.4.4 Crustaceans studies

The literature search for toxicity data of bisphenols and its alternatives to crustacean such as *Daphnia magna* and *Artemia species* was performed by combining the search terms "Daphnia magna" or "D. magna", "Artemia", "Artemia salina", "bisphenol" bisphenol A", "BPA", "BPA alternatives", "BPA analogues", "BPA substitutes", "toxicity", "ecotoxicity" with the Boolean operators "AND" or "OR". of a sequence of words that were associated with Boolean operators.

2.4.5 Nematoda studies

To obtain an overview of already conducted studies on BPA and BPA alternatives in the nematode *Caenorhabditis elegans* (*C. elegans*), an initial literature search was performed on the ZB MED Search Portal for Life Sciences LIVIVO. LIVIVO uses a semantic, index-based search engine and searches through library catalogues, publisher directories and other key life science databases such as MEDLINE (PubMed), AGRICOLA and AGRIS (www.livivo.de/app/misc/help/about, last accessed 16 Nov. 2023). The search was performed by combining the search terms "Caenorhabditis elegans" or "C. elegans", "nematodes", "bisphenol*" bisphenol a", "BPA", "BPA-alternative", "BPA-analogues", "BPA substitutes", "BPE", "BPS", "BPF", "BPZ", "TBBPA", "BPAP", "BPAF", "TCBPA", "toxicity", "neurotoxicity", "reproduction" with the Boolean operators "AND" or "OR". The LIVIVO search engine accounts automatically for synonyms, different word variations, abbreviations and translates key terms in multiple languages. The search results were automatically ranked by relevance (mainly according to frequency and position of search terms) and in a second step, manually prioritized based on the content of the abstracts. The total number of relevant hits was > 50 and therefore a subsequent manual perusal of all publications manageable. Relevant data and information on in particular toxicity, biological effects, sensitivity of life stages, mechanisms of toxicity and exposure conditions were extracted and evaluated for the present review.

2.4.6 Cnidaria studies

The literature search for toxicity data of bisphenols and its alternatives to freshwater cnidarians was performed through the use of a sequence of words that were associated with Boolean operators. The following word sequence was used and applied to All Fields in Web of Knowledge: ("bisphenol*" OR "BPA alternative") AND ("freshwater cnidaria" OR "hydrozoa" OR "hydroids" OR "hydra").

2.5 Studies with vertebrates

To collect the available data regarding the biological activity of BPA alternatives, we combined the following search strings joined by term "AND". For chemicals we searched for: i) general term bisphenol; ii) full chemical name (i.g. pergafast 201, bisphenol Z, bisphenol E, bisphenol P, bisphenol AP, bisphenol PH, bisphenol S, BPF); iii) their established abbreviations (i.e. BPZ, BPE, BPP, BPAP, BPPH BPS-MPE, BPS, BPPH, BADGE), or iv) their aliases (e.g. for BPS-MAE, we also searched for 4-((4-(Allyloxy)phenyl)sulfonyl) phenol, 4-(4-prop-2-enoxyphenyl)sulfonylphenol, 4-allyloxy-4'-hydroxy-diphenylsulfone)). Using the Web of Knowledge, we combined the chemical name with "fish" or "zebrafish" or "danio" or "amphibia" or "frog" or "xenopus". For specific endpoints, we also employed US EPA ECOTOX tool (https://cfpub.epa.gov/ecotox)[8], that is able to identify and visualize studies that focused on specific chemicals (bisphenols) and specific model organisms (e.g. fish models).

3. Occurrence of BPA alternatives in environmental compartments across Europe

In the last decades there have been growing concerns regarding the possible adverse effects of BPA to humans' and animals' health. These concerns, in addition to tight restrictions placed on BPA in many countries, have led to the development of alternative chemicals [e.g. bisphenol Z *(*BPZ), bisphenol E (BPE), bisphenol S-MAE (BPS-MAE), bisphenol P (BPP), bisphenol AP (BPAP), bisphenol B (BPB), bisphenol C (BPC), bisphenol S (BPS), bisphenol F (BPF), bisphenol AF (BPAF)]. As a result, these emerging environmental contaminants can now be found worldwide in various environmental compartments including water, sediment, sludge, soil, indoor dust and air. Furthermore, the use of BPA alternatives is expected to increase in the upcoming years due to proposals by EFSA and European Chemicals Agency (ECHA) to reduce the use of BPA and several other bisphenols with known adverse effects on humans and the environment.

Regarding their properties, most bisphenols are characterized as toxic (or very toxic) to aquatic organisms and have been shown to exhibit adverse effects on the endocrine, reproductive, metabolic and immune system in different species. Some of them are also persistent, mobile or bioaccumulative and are characterized as PBT/vPvB or PMT/vPvM. This information is summarized in a report regarding the "assessment of regulatory needs" published by ECHA in December 2021 [9].

Furthermore, considering the potential for widespread use of several bisphenols, the exposure of organisms to bisphenol mixtures in different environmental compartments (*i.e.,* surface and sea water, sediment, soil) is very likely to occur. Therefore, in the frame of this review, data regarding the occurrence of bisphenol mixtures (BPA and other bisphenols) in the above-mentioned environmental compartments were collected in order to identify the most frequently co-occurring bisphenols in the environment as well as their environmentally relevant concentrations.

3.1 BPA alternatives in freshwater

The occurrence of BPA and BPA alternatives in water samples has been investigated in several studies. Specifically, BPA, BPB, BPE and BPS were identified at levels up to 4.42, 3.66, 2.77 and 9.13 μg/L, respectively [10–13], while BPF, BPC, BPG, BPAF, BPM/BPP were identified at lower levels (up to 0.317, 0.012, 0.0209, 0.205, 0.06 μg/L, respectively) and BPZ, BPAP, BFDGE and TBBPA were not identified at detectable levels [11,14–18]. In addition, exceptionally high BADGE levels (*i.e.,* up to 28 μg/L) were identified in water samples collected from a lagoon in Campania in Southern Italy [12]. The levels of BPA and its alternatives in European waters are summarized in Fig.1 and Fig.2, respectively.

The detailed monitoring data retrieved by the identified studies are presented in the supplemental material S1.

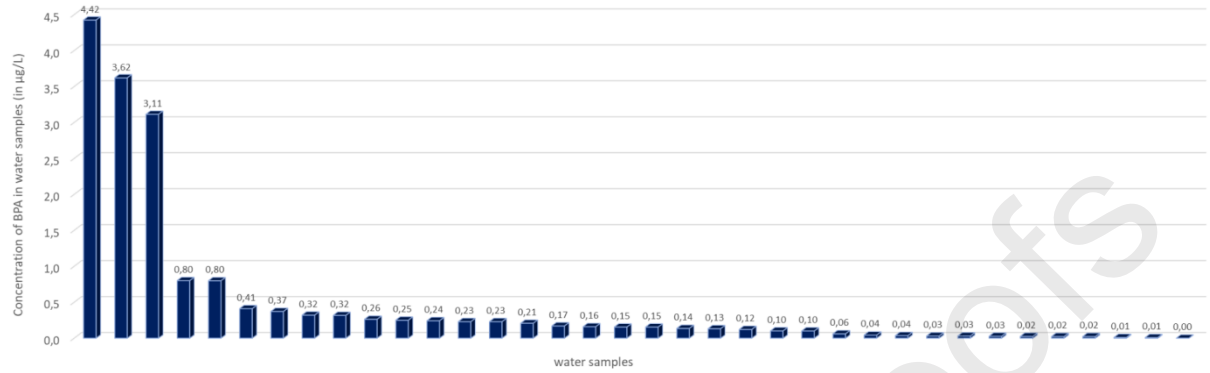

Fig. 1: Overview of the concentration levels of BPA in European water samples (rivers, estuaries, lakes, ponds and retention tanks). The detailed monitoring data retrieved by the identified studies are presented in the supplemental material S1. Values of 0.00 imply concentrations below the detection limit (LOD).

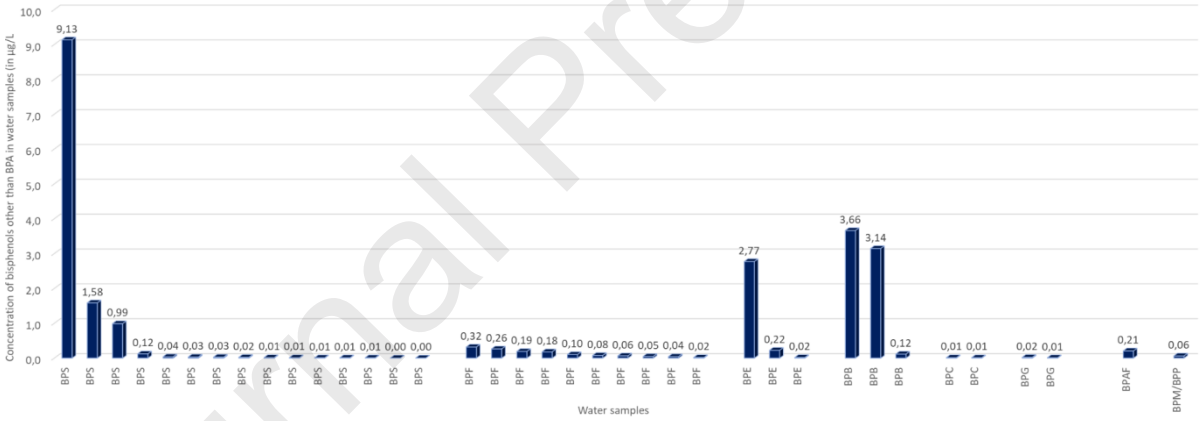

Fig. 2: Overview of the concentration levels of bisphenols (other than BPA) in European water samples (rivers, estuaries and lakes). The detailed monitoring data retrieved by the identified studies are presented in the supplemental material S1. Values of 0.00 imply concentrations below 0.004 µg/L.

3.2 BPA alternatives in sediment

In contrast to the water column, the occurrence of BPA and BPA alternatives in sediment samples has been less investigated so far. In sediment samples, BPA was found at higher levels than all other detected bisphenols. BPA was detected in countries across Europe (Germany, Italy, Portugal and the UK) at levels up to 190 ng/g dw [11,12,15,19]. BPAF and BADGE were also detected at high levels (up to 155 and 61 ng/g dw respectively) in sediment samples collected from a lagoon in Campania in Southern Italy, while BPAF was also detected in samples collected from Portugal [11,12]. BPS, BPB and BPF were detected at significant levels in different countries (Portugal, UK, Italy and

Germany), while other bisphenols such as BPZ, BPAP, BPE and BPP were not identified at detectable levels [11,12,15,17,19]. The levels of BPA and its alternatives in sediment are summarized in Figure 3. The detailed monitoring data retrieved by the identified studies are presented in the supplemental material S1.

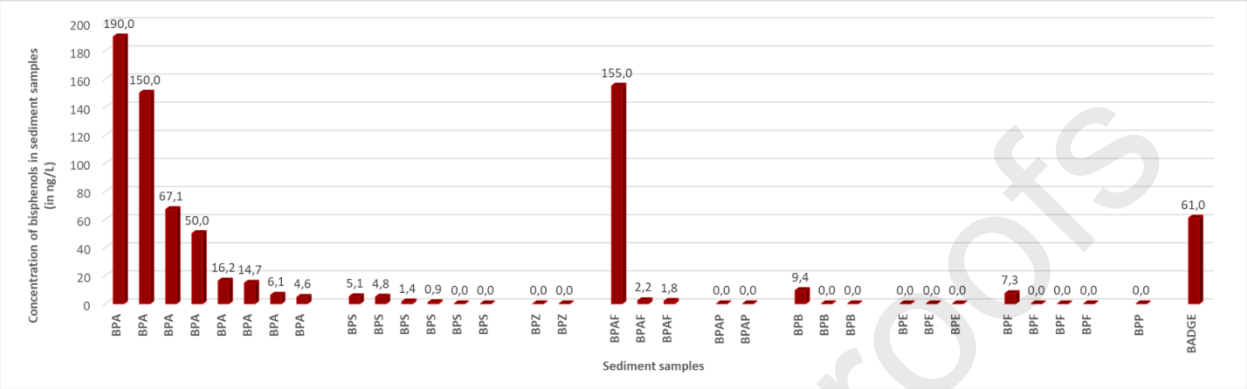

Fig.3: Overview of the concentration levels of BPA and its alternatives in the sediment samples across Europe. The detailed monitoring data retrieved by the identified studies are presented in the supplemental material S1. Values of 0.00 imply concentrations below the detection limit (LOD).

3.3 BPA alternatives in terrestrial ecosystems

Bisphenol based substances can occur in agricultural soils due to practices of soil amendment with biosolids or irrigation with wastewater. In agricultural soils, BPA concentrations range between 0.55 and 147 µg/kg dry weight [20]. Only a limited number of studies have investigated levels of BPA alternatives in terrestrial samples. So far, one unique study has been identified reporting the analysis of various bisphenols in environmental samples from soil [21]. This study was conducted in Spain and a comparison of the levels of bisphenols identified in soil samples from agricultural and industrial areas was carried out. The results revealed that only BPA and BPF were identified at levels 17.2 -126.2 and 4.8 – 127.2 ng/d dw, respectively, in soil samples from industrial areas, and 1.1 – 55.9 and up to 15.3 ng/d dw in soil samples from agricultural areas (see supplementary material S1). All other analyzed bisphenols (BPB, BPP, BPS, BPZ, BPAF, BPAB) were not found at detectable levels.

3.4. Conclusion

BPA and BPA alternatives are ubiquitous chemicals found in all studied environmental compartments. Although BPA is currently regulated and its use is restricted in many products, its levels continue to dominate in comparison with its alternatives in environmental samples of water and sediment. The broad use of BPA and its alternatives in consumer products has led to its widespread distribution in aquatic and terrestrial ecosystems, where it can accumulate and persist for extended periods. Similar to BPA, its alternatives have been detected in environmental samples, raising questions about their potential health and ecological impacts. Therefore, the future monitoring and surveillance of water, sediment and soil quality should be enhanced to include measurements of BPA alternatives to monitor their levels and ensure development of timely mitigation strategies. The fact that only a limited number of monitoring studies for bisphenols other than BPA were identified to be

conducted in Europe, even though their use is constantly increasing as they are used as alternatives to BPA, reinforce the need for further and extended monitoring activities in different environmental compartments. Finally, further study is needed in order to investigate the possible adverse effects of bisphenols on organisms belonging in different taxa when they will be exposed to environmentally relevant concentrations (as revealed by monitoring studies) of mixtures of bisphenols. The results of this "retrospective risk assessment" could be exploited to highlight the possible need for mitigation strategies at European level.

Considering the different emissions of bisphenols, from dental treatment, pesticides, thermal papers, food containers, lacquers or paints, their co-occurrence with analogues or other substances is likely, leading to several challenges regarding waste treatment, fate into environmental compartments and consequent effects.

4. Predictive models, and bioactivity of BPA Alternatives

Understanding the toxicity of BPA and its alternatives is of paramount importance due to their widespread use and presence in the environment. The ecotoxicity of BPA alternatives has become a subject of increasing concern. As these alternatives, such as BPS and BPF, are being widely used as replacements for BPA, it is crucial to assess their potential impact on the environment. Ecotoxicity studies aim to evaluate the effects of these alternatives on organisms, including both the invertebrates and vertebrates. Understanding the ecotoxicity of BPA alternatives is essential for identifying any potential risks they may pose to aquatic life, ecological balance, and overall environmental health. It provides valuable insights for regulators, policymakers, and manufacturers to make informed decisions that prioritize the development and use of safer alternatives, ultimately safeguarding our ecosystems and biodiversity. With respect to priorities in PARC, collected information regarding the bioactivity of BPA alternatives will form a basis for selection of suitable BPA alternatives that will serve as model chemicals for development of new approach methodologies (NAMs). With respect to NAMs, *in silico* approaches have emerged as valuable tools in the evaluation of ecotoxicity, offering efficient and cost-effective tools for predicting and assessing the potential ecological impact of chemicals. By utilizing computer models and algorithms, *in silico* approaches enable the analysis of large chemical datasets, predicting various ecotoxicological endpoints, such as toxicity to aquatic organisms or bioaccumulation potential. These methods leverage available data on chemical structure, physicochemical properties, and biological activity to generate predictive models and provide valuable insights into the potential hazards of chemicals. Within this chapter, we have provided a comprehensive summary of the current advancements in both *in silico* approaches and *in vivo* models utilized for screening, prioritization, and toxicological evaluation of these chemicals.

4.1. The role of physicochemical properties for the toxicity analysis of BPA alternatives

The hydrophobicity of BPA alternatives expressed as logK_{ow} of the neutral species ranges from 1.3 < logK_{ow} < 7.15 (Fig.4a) and covers almost six orders of magnitude. BPA itself has an experimental logK_{ow} of 3.32, which is very close to the median of the distribution of 3.64. Few experimental logK_{ow} values were available for the BPA alternatives. The common prediction models (ACD, OPERA, Episuite) yielded estimates that varied up to two orders of magnitude magnitude (Supp.info 3, Table SI-1). In Tab.2 and Fig.4a the mean of the predictions was included as no preference could be given to any of the prediction models. As hydrophobicity is one main driver of toxicity, it is vital that more reliable logK_{ow} become available, ideally experimental values.

BPA is a diprotic acid due to the two phenolic groups with pK_a-values of 9.7 and 10.5, which means that BPA is present in its neutral form at pH 7.4 ($\alpha_{neutral}$ (pH 7.4) = 1), which is the pH in standard cell assays. BPS is much more acidic due to the sulfonyl group and is 49% negatively charged at pH 7.4 and 15% is even double negatively charged (Tab. 2). TCBPA and TBBPA have similar speciation but are located at the other end of the hydrophobicity scale (logK_{ow} 6.19 and 6.99 (Fig.4)). Most of the BPA alternatives have one or two phenolic groups but almost none are deprotonated at pH 7.4. In some cases, the phenols are substituted by ethers (e.g., BADGE, Table 1), and these alternatives are also neutral. BTUM is a special case as it does not contain any phenolic groups but two sulfonamide groups, which are N-acidic groups, with pK_a-values of 4.9 and 4.6, which means that BTUM is double deprotonated and anionic at pH 7.4 (Table 1). BPSMPE4 has only one phenolic group but has a higher acidity of this phenolic group (lower pK_a) due to the electron-withdrawal from the sulfonyl group and is therefore almost completely anionic (α_{anion} (pH 7.4) = 0.93).

In summary, both, the logK_{ow} and pK_a values of the BPA alternatives vary over 6 to 10 log-units (Fig. 4a), which means that already based on the physicochemical properties we can expect a high diversity in environmental fate and toxicity. This does not even consider the stability of the BPA alternatives, which in addition needs to be considered.

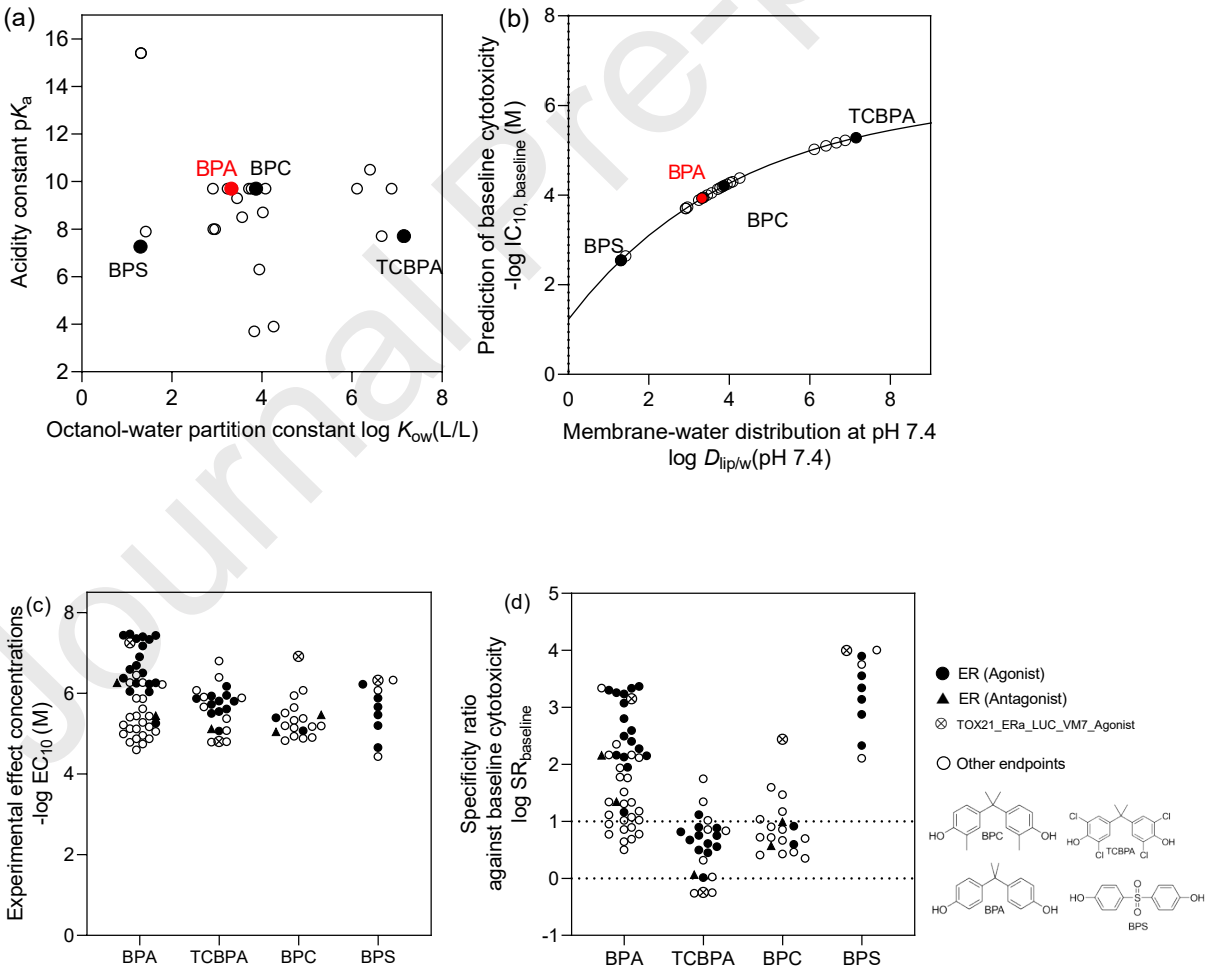

Fig.4: The main physicochemical properties of BPA alternatives: **(a)** Octanol-water partition constant of the neutral species logK_{ow} plotted against the acidity constants pK_a; **(b)** Baseline toxicity QSAR

for generic cell lines [22] and predictions of baseline cytotoxicity inhibitory concentrations IC10 baseline for all BPA alternatives (Tab.2); **(c)** Experimental EC_{10} for BPA and three exemplary BPA alternatives in the Tox21 cell-based bioassays (data in Supp.info 3, Table S2). **(d)** Specificity ratios $SR_{baseline}$ calculated from the $IC_{10,\ baseline}$ (Supp. info, Table S3) and the EC10 in Fig. 4c (data in Supp. info 3, Table S3).

4.2. Prediction of baseline toxicity of BPA alternatives

As many BPA alternatives are deprotonated, their affinity to biological membranes is not directly correlated to the logK_{ow}. Baseline toxicity prediction models for partially or fully charged chemicals have typically the affinity to biological membranes expressed as logD_{lipw} as hydrophobicity descriptor [7]. Fig.4b depicts a generic baseline toxicity model for human cell assays (eq. 4) developed by Lee et al. 2021 [22]. The predicted baseline cytotoxicity $IC_{10,baseline}$ ranged from 5.6 µM for TBBPA to 2.2 mM for BPS (Tab.2). BPA and BPC lie in the middle with $IC_{10,baseline}$ of 79 and 34 µM, respectively. Overall, the potency predicted with $IC_{10,baseline}$ already ranges by a factor of 386 between BPA and its alternatives.

4.3. *In vitro* cell-based bioassays for mode-of-action analysis

Experimental reporter gene assays data from Tox21 were available for BPA and 15 BPA alternatives (Table S2). The distribution of experimental effect data is depicted in Figure 1c on the example of BPS, BPA, BPC and TCBPA. On first view, BPS, TCBPA, BPA and BPC appear having similar potency in the micromolar effect concentration range (Figure 1c), but if the EC_{10} are compared to the associated predicted baseline toxicity ($SR_{baseline}$) distinct differences become apparent (Figure 1d).

The $SR_{baseline}$ (Figure 1d) is clearly highest for both, BPA and BPS. BPA was tested highly specific in bioassays indicative of the activation of the estrogen receptor (ER) with $SR_{baseline}$ up to 2300. Despite its lower overall potency, the $SR_{baseline}$ of BPS was up to 4 times higher than of BPA. This means that with respect to its intrinsic effect BPS is rather similar to BPA even if estrogen-specific effects are only triggered at higher nominal concentrations due to the lower hydrophobicity of BPS. This conclusion needs to be treated with certain caution due to the high variability of hydrophobicity prediction models for BPS. A SR of 10 represents the threshold between specific and highly specific effects Fig.4d [23]. A compound activating a response below 10 is considered behaving as baseline toxicant. TCBPA is of similar potency expressed as EC_{10} as BPA Fig.4c), however, it has a $SR_{baseline}$ below 10 in most assays in our dataset. Therefore, we can conclude it is acting like a baseline toxicant meaning that TCBPA activates the ER but at concentrations where nonspecific toxicity occurs.

The black filled circles in Fig.4d are endpoints related to activation of the ER or indirect effects on ER. ER-related endpoints are most active with highest $SR_{baseline}$ for BPA and BPS but are at $SR_{baseline}$ <10 for TCBPA and BPC. The TOX21_ERa_LUC_VM7 assay (formerly called TOX21_ERa_LUC_BG1, [24] was identified as the most sensitive estrogen receptor reporter gene assay [25] and is highlighted in Fig.4c and d with a cross through the circle. It is indeed also one of the most sensitive assays for BPA alternatives with exception of the TCBPA, which confirms that the bulky TCBPA is a poor activator of ER, which had already been shown previously [26]. The activation of nuclear receptors with $SR_{baseline}$ close to 1 is likely to be an artifact and caused by the cytotoxicity burst. The term “cytotoxicity burst” describes the phenomenon of enhanced non- specific activation of stress responses and even nuclear receptor binding that occur close to cell death [23,27,28].

It is somewhat puzzling on first sight that also ER antagonism seems to play a role at rather low concentration (Fig.4c). However, the $SR_{baseline}$ analysis clearly indicates that this effect is not specific and is likely to also be an artifact of the cytotoxicity burst (Fig.4d).With this knowledge in mind, we can interpret the effect data in the *in vitro* assays from a different viewpoint: Specific effects matter more if they have a $SR_{baseline}$ > 10. Apart from the large number of estrogenicity assays with $SR_{baseline}$ > 10, the antagonistic mode of androgen receptor (AR) assays were also active with $SR_{baseline}$ of 33 for BPA, 39 for BPC and 128 for BPS. Activation of ER is commonly accompanied by antagonism of AR [26].

The vitamin D receptor (assay ATG_VDRE_CIS_up) was activated with a $SR_{baseline}$ of 22 by BPA, 8 by BPC and 5625 by BPS. The pregnane X receptor (PXR) and constitutive androstane receptor (CAR), both of which are regulators of xenobiotic metabolism, were also activated by BPA but not by any other BPA alternatives. Most notably, the TOX21_MMP assays (MMP stands for mitochondrial membrane potential, [29] also captured the specific mitochondrial toxicity of BPA alternatives. BPA is known to exert mitochondrial toxicity [30], and the $SR_{baseline}$ of 60 by BPA, 56 by TCBPA and 15 by BPC (Supp.info 3, Table SI-3) indicate that these analogs are also mitochondrial toxicants, while BPS was not active in TOX21_MMP (Supp.info 3, Table SI-2).

For the analysis in Fig.4c and 4d we just selected BPA and 3 of the 15 BPA alternatives, where we found data for *in vitro* bioassays to illustrate important differences. An overview about the analysis of the effects of all BPA alternatives in *in vitro* reporter gene assays is given in the Supplementary information.

Cytotoxicity data is rather scarce in the in vitro database and available only for 14 compounds and fewer assays (Supp. info 2, Fig.SI-2). Of the reported IC_{10} for cytotoxicity, BPSMPE4 and TBBPA have the highest TR >1000 and BCP, BPAB, BzPB, BPA, BPZ, BPPH, TCBPA and BCAF were mere baseline toxicants with TR <10 (Supp. info 2, Fig.SI-2). Only for three chemicals (BPZ, BPPH, BPC), both receptor-activation and cytotoxicity were available. Therefore, no direct comparison was possible for the larger chemical set, and we analyzed cytotoxicity (Supp. info 2, Fig. SI-2) and reporter gene activation (Supp. info 2, Fig. SI-3) independently.

BPA was one of the most active chemicals (but also one that was tested in most bioassays) but the $SR_{baseline}$ was in most assays higher for BPS than BPA. Some alternatives, especially the hydrophobic ones (BPPH, TCBPA, BPSMPE4) often had lower $SR_{baseline}$, despite overall rather high potency. This analysis clearly demonstrated the importance to evaluate not only the absolute potency differences but consider the potency also in relation to the degree ($SR_{baseline}$) and type of specificity (affected endpoint). To differentiate between specific and non-specific effects is vital for risk assessment [28] because non-specific effects will occur for all endpoints, albeit at high nominal concentrations and can be confidently predicted by baseline toxicity models across cells but also all across aquatic species. To know which specific effects are relevant is also important. BPA and many of its alternatives are estrogenic but we also find other relevant endpoints related to xenobiotic metabolism and mitochondrial toxicity, which can guide ecotoxicity testing.

Tab.2. Chemical identifiers and physicochemical properties of the BPA alternatives. Octanol-water partition constant of the neutral species, $\log K_{ow}$; acidity constant pK_a. Thereof derived speciation and liposome-water distribution ratios $\log D_{lipw}$(pH 7.4). $IC_{10,baseline}$ predicted with the baseline toxicity QSAR from Lee et al. 2021 [22]. The literatures source and the detailed predictions for $\log K_{ow}$ are given in the Supp.ninfo 3, Table SI-1.

IUPAC-Name	Abbreviation	Short abbreviation	MW (g/mol)	CASRN	logK_{ow} [L/L]	pK_a (acid)	pK_a (acid)	Fraction neutral species (pH 7.4)	log D_{lipw} (pH 7.4)	$IC_{10,baseline}$ (M)
4,4'-Sulfonyldiphenol	Bisphenol S	BPS	250.27	80-09-1	1.30	7.26	7.92	36%	1.05	4.74E-03
2,2,4,4-Tetramethyl-1,3-cyclobutanediol (racemate)	r-CBDO	rCBDO	144.21	3010-96-6	1.30	15.4	-	100%	1.43	2.21E-03
cis-2,2,4,4-Tetramethyl-1,3-cyclobutanediol	c-CBDO	cCBDO	144.21	3039-96-1	1.30	15.4	-	100%	1.43	2.21E-03
2,4'-Dihydroxydiphenyl sulfone	2,4-BPS	24BPS	250.27	5397-34-2	1.42	7.9	9.1	76%	1.44	2.17E-03
Benzyl 4-hydroxybenzoate	Benzylparaben	BzPB	228.25	94-18-8	3.56	8.5	-	93%	3.69	7.63E-05
4-[[4-(Allyloxy)phenyl]sulfonyl]phenol	4-APS	4APS	290.33	97042-18-7	2.92	8	-	80%	2.98	1.81E-04
N-(p-toluenesulfonyl)-N'-(3-(p-toluenesulfonyloxy)phenyl)urea	Pergafast 201	Pergafast	460.52	232938-43-1	4.19	3.70	-	0%	3.35	1.13E-04

4-((4-Isopropoxyphenyl)sulfonyl)phenol	D-8	D8	292.35	95235-30-6	2.96	8.00	-	80%	3.02	1.73E-04
Bis(4-hydroxyphenyl)methane	Bisphenol F	BPF	200.23	620-92-8	2.91	9.7	10.50	100%	3.06	1.64E-04
4-[4-(benzyloxy)benzenesulfonyl]phenol	BPS-MPE4	BPSMPE4	340.39	63134-33-8	4.18	6.3	-	7%	3.56	8.82E-05
1,1-Bis(4-hydroxyphenyl)ethane	Bisphenol E	BPE	214.26	2081-08-5	3.12	9.7	10.50	100%	3.27	1.25E-04
4,4'-Bis(p-tolylsulfonylureido)diphenylmethane	BTUM	BTUM	592.69	151882-81-4	5.21	3.90	4.60	0%	4.38	3.69E-05
4,4'-Thiodiphenol	BPT	BPT	218.27	2664-63-3	3.34	9.7	10.50	100%	3.49	9.56E-05
5,5-Dimethyl-3,7-dioxa-1,9(2)-bis(oxirana)-4,6(2,4)-dibenzenanonaphane	BADGE	BADGE	340.42	1675-54-3	3.71	-	-	100%	3.86	6.28E-05
Benzeneacetic acid, 4-hydroxy-alpha-(4-hydroxyphenyl)-, methyl ester	Bz	Bz	258.27	5129-00-0	2.73	9.3	10.10	99%	2.87	2.11E-04
4,4'-(Propane-2,2-diyl)diphenol	Bisphenol A	BPA	228.29	80-05-7	3.32	9.7	10.5	100%	3.65	7.95E-05

4,4'-(1-Phenylethylidene)bisphenol	Bisphenol AP	BPAP	290.36	1571-75-1	4.43	9.70	10.50	100%	4.59	3.04E-05
4,4'-(Butane-2,2-diyl)diphenol	Bisphenol B	BPB	242.32	77-40-7	3.95	9.7	10.50	100%	4.10	4.87E-05
3,3'-Dimethylbisphenol A	Bisphenol C	BPC	256.35	79-97-0	4.32	9.7	10.5	100%	4.48	3.35E-05
4,4'-(1,1,1,3,3,3-Hexafluoropropane-2,2-diyl)diphenol	Bisphenol AF	BPAF	336.23	1478-61-1	3.70	8.7	9.50	95%	3.83	6.48E-05
1,1'-Bis(4-hydroxyphenyl)cyclohexane	Bisphenol Z	BPZ	268.36	843-55-0	4.54	9.7	10.50	100%	4.70	2.74E-05
4,4'-[1,4-Phenylenebis(1-methylethylidene)] bis-phenol	Bisphenol P	BPP	346.47	2167-51-3	6.10	9.7	10.30	100%	6.28	8.50E-06
2,2-Bis(4-hydroxy-3-isopropylphenyl)propane	Bisphenol G	BPG	312.45	127-54-8	6.04	10.5	11.3	100%	6.22	8.79E-06
3,3',5,5'-Tetrabromobisphenol A	TBBPA	TBBPA	543.88	79-94-7	6.99	7.7	8.50	65%	7.01	5.62E-06
2,2-Bis(2-hydroxy-5-biphenylyl)propane	BisOPP-A	BPPH	380.49	24038-68-4	6.59	9.7	10.5	100%	6.77	6.39E-06

2,2',6,6'-Tetrachlorobisphenol A	TCBPA	TCBPA	366.06	79-95-8	6.19	7.7	8.50	65%	6.21	8.87E-06

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4.4. Predictive models for ecotoxicity of bisphenols alternatives

Two phases can be distinguished in the dose-response relationship of a chemical in an organism: the toxicokinetic (TK), that is the fate of the compound in the organism including absorption, distribution, metabolism and excretion (ADME), and the toxicodynamics (TD), that is the expression of the toxicity/effect of the substance at the site of action.

The simplest approaches to predict TK or TD are based on steady-state assumptions (e.g. quantitative structure-relationship models (QSAR)) which reflect the equilibrium between accumulation of a compound and its elimination from all exposure routes. In some cases, the steady-state assumption may not hold, particularly in environmental conditions under which chemical occurrence and exposure may vary considerably.

Therefore, in order to review the models developed to predict TK and TD of bisphenol derivatives, we structured this chapter in two main parts: the first part presents static models assuming a steady state and the second part presents dynamic models (i.e., those where temporal variation of exposure or effects is explicitly taken into account). Further, commercially available software TEST, VEGA, OPERA, ECOSAR, and the OECD QSAR toolbox were checked for the availability of implemented predictive models for estimating the ecotoxicity of compounds. A list of all the available models that incorporated either BPA or BPA alternatives in their training set was compiled (Supp. info 4, Tab.S2.1). Moreover, we carried out a comparative analysis of the predictions between the VEGA and TEST software and checked how the predictions differed within one software.

4.4.1. QSAR model: models assuming steady state

Currently, many available scientific papers are focused on QSAR models for predicting toxicity for a wide group of compounds, such as contaminants of emerging concern (CEC) [31] or ionizable organic chemicals (IOC) [32]. In contrast, no specific models for predicting ecotoxicological properties have been dedicated only to bisphenols and their derivatives.

Usually, available models are focused on compounds from the different chemical groups and contain in the training set only single bisphenols (e.g., BPA, BPAF, and BPS). The variety of relevant ecotoxicological endpoints in published models is also severely limited and is not always consistent with risk assessment requirements. Supp. info 4 (Tab. S2.1) presents gathered available models developed for predicting ecotoxicological endpoints containing bisphenols and their derivatives in the training set. There are models that focus on establishing the ecotoxicity for different species separately (e.g. *Danio rerio and Ozyrias latipes*), but also models that cover simultaneously different taxonomic groups (e.g algae, daphnid, and fish) – multispecies models. The most often included in the training set are BPA, TBBPA, and BPF. Each of these models could be applied for predicting the ecotoxicity of bisphenols derivatives, however, in practice, it requires evaluating if the model is scientifically valid and fulfills OECD recommendations for developing and validating the QSAR models. Moreover, the introduction of the properly derived structure of the compounds (molecular descriptors used in the models) and assessing if obtained predictions are within the space of the applicability domains (AD) of the models are needed, which requires specialized knowledge in the field of computational chemistry.

4.4.2. Comparison of available tools for predicting ecotoxicity of bisphenols

The other source of predictive models available within the public domain is open-source tools, like e.g., TEST, VEGA, OPERA and the OECD QSAR toolbox. These tools have implemented different models that can be used for estimating the ecotoxicity of compounds (Supp. info 4, Tab. S2.1 and S2.2). Predictions of an endpoint values are based here on the structural similarity between the compounds of interest and a training set on which the model was developed. In some cases, it may be that the predictive ability of such models for the chemicals of interest is not appropriate, and predictions can fall outside the model's applicability domain (AD), making them less reliable. Further, in each of these software tools, implemented models were developed using different compounds for training and validation, even within the same endpoint.

In Task 5.2.2 of PARC there was a need to verify whether the available tools could be used to predict the ecotoxicity of BPA alternatives. In the first step, the acute toxicity of *Daphnia magna* was analyzed. Two models for estimating EC_{50} were available in the VEGA. The predictions for 16 BPA alternatives established in PARC derived from the VEGA tool were analyzed and compared to the experimental data whenever possible (Tab.3). Here, the 'prediction' and 'reliability' were considered. 'Prediction' means how well the model predicted the result, relative to the training set. If there is a good prediction, it means that there were chemical compounds in the training set with a similar structure to the predicted compound. In contrast, 'reliability' refers to the validation of the predictions made, i.e. whether the prediction is reliable, or more precisely whether it is within the AD. Almost all prediction results obtained using two different acute toxicity models for *Daphnia magna* 48h (EC_{50}) were found to be both not very accurate, due to the lack of similar molecules in the training set, and unreliable (out of AD). The exception is one prediction made for BPZ (CAS: 843-55-0) based on experimental data implemented in the software. This means that by possessing experimental ecotoxicity data for the group of compounds in question, accurate and reliable predictions can be made, but currently the detailed data necessary are mostly lacking.

Table 3. Comparison of the acute toxicity (*Daphnia magna*) predictions for 16 bisphenols alternatives using VEGA software [33,34].

IUPAC – Compound Name	***Molecule CAS***	***Experimental data**** ***EC_{50}*** ***[mg/L]***	***VEGA***					
			Daphnia magna Acute (EC_{50}) Toxicity model (IRFMN) Predicted [mg/L]			***Daphnia magna Acute (EC_{50}) Toxicity model (IRFMN/Combase) Predicted [mg/L]***		
2,2-Bis(2-hydroxy-5-biphenylyl)propane	*24038-68-4*	*N/A*	*0.4695*	*P*	*R*	*0.0769*	*P*	*R*

4-[4-(benzyloxy)benzenesulfonyl]phenol	*63134-33-8*	*N/A*	*15.02*	*P*	*R*	*0.1522*	*P*	*R*
4-((4-Isopropoxyphenyl)sulfonyl)phenol	*95235-30-6*	*N/A*	*20.07*	*P*	*R*	*0.1045*	*P*	*R*
4-[[4-(Allyloxy)phenyl]sulfonyl]phenol	*97042-18-7*	*13.5*	*22.01*	*P*	*R*	*0.0702*	*P*	*R*
4,4'-(1-Phenylethylidene)bisphenol	*1571-75-1*	*N/A*	*6.76*	*P*	*R*	*0.0333*	*P*	*R*
1,1-Bis(4-hydroxyphenyl)ethane	*2081-08-5*	*N/A*	*16.07*	*P*	*R*	*0.1688*	*P*	*R*
4,4'-[1,4-Phenylenebis(1-methylethylidene)] bis-phenol	*2167-51-3*	*N/A*	*0.8283*	*P*	*R*	*0.0056*	*P*	*R*
1,1'-Bis(4-hydroxyphenyl)cyclohexane	*843-55-0*	*1.8*	*6.11*	*P*	*R*	*0.0091*	*P*	*R*
Benzyl 4-hydroxybenzoate	*94-18-8*	*6*	*1.58*	*P*	*R*	*2.05*	*P*	*R*
2,2,4,4-Tetramethyl-1,3-cyclobutanediol (racemate)	*3010-96-6*	*106*	*85.02*	*P*	*R*	*1.48*	*P*	*R*
Benzeneacetic acid, 4-hydroxy-alpha-(4-hydroxyphenyl)-, methyl ester	*5129-00-0*	*N/A*	*28.9*	*P*	*R*	*0.27*	*P*	*R*
4,4'-Thiodiphenol	*2664-63-3*	*N/A*	*34.63*	*P*	*R*	*0.0553*	*P*	*R*
N-(p-toluenesulfonyl)-N'-(3-(p-toluenesulfonyloxy)phenyl)urea	*232938-43-1*	*56*	*0.0022*	*P*	*R*	*0.0488*	*P*	*R*
4,4'-Bis(p-tolylsulfonylureido)diphenylmethane	*151882-81-4*	*N/A*	*0.2701*	*P*	*R*	*1.05*	*P*	*R*

2,4'-Dihydroxydiphenyl sulfone	*5397-34-2*	*N/A*	*10.48*	*P*	*R*	*0.6772*	*P*	*R*
2,2',6,6'-Tetrachlorobisphenol A	*79-95-8*	*N/A*	*0.5831*	*P*	*R*	*0.0015*	*P*	*R*

*Experimental data were collected using QSAR TOOLBOX,

P - Prediction: Good (Green color), Unfavorable (Red color),

R - Reliability of the prediction (AD): Green (In AD), Orange (Possibly Out of AD), Red (Out of AD).

In the next step, LC_{50} values for *Daphnia magna* predicted using TEST and VEGA software were analyzed (Tab.4). There are apparent differences between the predictions obtained for acute toxicity, depending on the model and software used. In the TEST software for each modelling method, good predictions were obtained for two compounds, BPAP (Bisphenol AP, CAS: 1571-75-1) and BPE (CAS: 2081-08-5). This means that the training sets used in the models contained structurally similar compounds. Between themselves, these compounds are also similar, differing only in the substitution of the central carbon atom. In BPE, the hydrogen atom on the central carbon atom was substituted with benzene in the case of Bisphenol AP. On the other hand, applying the VEGA the predictions for the previously mentioned bisphenol pair are incorrect and out of the AD. This may mean that for both models presented in the VEGA software, there are no structurally similar compounds to BPAP and BPE used for training the models. In contrast, the models considered in VEGA contained chemical compounds similar to the three compounds considered as alternatives: 2,2,4,4-Tetramethylcyclobutane-1,3-diol (CAS: 3010-96-6), 4,4'-Thiodiphenol (CAS: 2664-63-3) and 2,4'-Dihydroxydiphenyl sulfone (CAS: 5397-34-2). In each of the models used, regardless of the software (with a few exceptions), the predictions obtained do not fall within the AD. This is a result of the lack of bisphenols in the training sets, as well as the lack of experimental data for compounds similar to bisphenols.

Summarizing, available tools for predicting activity/physicochemical properties are beneficial in the primary assessment of chemicals, however, they do not cover BPA alternatives in case of acute toxicity to *Daphnia magna*. Evaluation of the applicability of other ecotoxicity models implemented in those tools in terms of their reliability for bisphenols alternatives alternatives were recently conducted in Lagares et al. 2023 [35]. However, it is already evident that there is a very high demand for *in silico* methods covering the whole group of bisphenols and their derivatives (also including bisphenol alternatives) with regard to the endpoints importance also from the regulatory point of view.

98 **Table 4.** Predictions made in selected software for bisphenol alternatives [33,34].

99

IUPAC – Compound Name	*Molecule [CAS]*	*Experimental Value*	*TEST Daphnia magna LC_{50} (48h) [-log10(mol/L)]*													*VEGA Daphnia magna LC_{50} 48h Predicted [-log(mol/L)]*					
		LC_{50} - log10(mol/L)	*Consensus Model*			*Hierarchical Clustering*			*Single Model*			*Group Contribution Model*			*Nearest Neighbor Model*			*(EPA)*			*(DEMETRA)*		
2,2-Bis(2-hydroxy-5-biphenylyl)propane	*24038-68-4*	*N/A*	*6.99*	*P*	*R*	*7*	*P*	*R*	*7.2*	*P*	*R*	*7.97*	*P*	*R*	*5.81*	*P*	*R*	*5.55*	*P*	*R*	*4.5*	*P*	*R*
4-[4-(benzyloxy)benzenesulfonyl]phenol	*63134-33-8*	*N/A*	*5.37*	*P*	*R*	*5.62*	*P*	*R*	*5.86*	*P*	*R*	*4.98*	*P*	*R*	*5.01*	*P*	*R*	*4.88*	*P*	*R*	*4.72*	*P*	*R*
4-((4-Isopropoxyphenyl)sulfonyl)phenol	*95235-30-6*	*N/A*	*4.74*	*P*	*R*	*4.79*	*P*	*R*	*5.41*	*P*	*R*	*4.32*	*P*	*R*	*4.45*	*P*	*R*	*4.46*	*P*	*R*	*4.03*	*P*	*R*
4-[[4-(Allyloxy)phenyl]sulfonyl]phenol	*97042-18-7*	*N/A*	*4.99*	*P*	*R*	*4.94*	*P*	*R*	*5.31*	*P*	*R*	*4.72*	*P*	*R*	*N/A*	*P*	*R*	*4.4*	*P*	*R*	*4.08*	*P*	*R*

4,4'-(1-Phenylethylidene)bisphenol	1571-75-1	N/A	5.45	P	R	5.6	P	R	5.75	P	R	5.09	P	R	5.34	P	R	4.67	P	R	3.74	P	R
1,1-Bis(4-hydroxyphenyl)ethane	2081-08-5	N/A	4.8	P	R	4.6	P	R	4.96	P	R	5.43	P	R	4.25	P	R	3.66	P	R	3.46	P	R
4,4'-[1,4-Phenylenebis(1-methylethylidene)] bis-phenol	2167-51-3	N/A	5.82	P	R	6.32	P	R	6.75	P	R	5.55	P	R	4.65	P	R	5.35	P	R	4.4	P	R
1,1'-Bis(4-hydroxyphenyl)cyclohexane	843-55-0	N/A	5.18	P	R	5.45	P	R	4.99	P	R	4.92	P	R	5.34	P	R	4.3	P	R	3.6	P	R
Benzyl 4-hydroxybenzoate	94-18-8	N/A	4.87	P	R	5.07	P	R	5.09	P	R	4.77	P	R	4.56	P	R	4.76	P	R	4.68	P	R
2,2,4,4-Tetramethyl-1,3-cyclobutanediol (racemate)	3010-96-6	N/A	2.87	P	R	1.96	P	R	3.42	P	R	3.23	P	R	N/A	P	R	2.53	P	R	4.09	P	R
Benzeneacetic acid, 4-hydroxy-alpha-(4-hydroxyphenyl)-, methyl ester	5129-00-0	N/A	4.81	P	R	4.04	P	R	4.89	P	R	5.44	P	R	4.89	P	R	3.86	P	R	3.54	P	R
4,4'-Thiodiphenol	2664-	N/A	N/	P	R	N/	P	R	N/	P	R	N/	P	R	4.7	P	R	2.2	P	R	3.	P	R

	63-3		A			A			A			A			8			9			9		
N-(p-toluenesulfonyl)-N'-(3-(p-toluenesulfonyloxy)phenyl)urea	232938-43-1	N/A	N/A	P	R	N/A	P	R	N/A	P	R	N/A	P	R	4.52	P	R	5.13	P	R	6.11	P	R
4,4'-Bis(p-tolylsulfonylureido)diphenylmethane	151882-81-4	N/A	N/A	P	R	N/A	P	R	N/A	P	R	N/A	P	R	4.66	P	R	6.26	P	R	6.97	P	R
2,4'-Dihydroxydiphenyl sulfone	5397-34-2	N/A	4.61	P	R	4.54	P	R	5.15	P	R	4.3	P	R	4.45	P	R	2.6	P	R	3.06	P	R
2,2',6,6'-Tetrachlorobisphenol A	79-95-8	N/A	6.55	P	R	7.03	P	R	6.96	P	R	6.77	P	R	4.99	P	R	4.8	P	R	3.83	P	R

100 P - Prediction: Reliable (Green color), Unreliable (Red color),

101 R - Reliability of the prediction (AD): Green (In AD), Orange (Possibly Out of AD), Red (Out of AD).

4.4.3. Dynamic models

Toxicokinetic models

Dynamic toxicokinetic models were developed to support NAM using zebrafish eleutheroembryo [36]. Indeed, analytical methods are still missing to measure organ concentrations. Therefore, physiologically based toxicokinetic (PBTK) modelling may overcome current limitations to help understand the relationship between toxic effects and internal exposure in various organs. A model was specifically developed to simulate the toxicokinetics of BPA, BPAF, BPF, and BPS through the eleutheroembryo tissues while considering the body and organ growth [37]. This model was developed using data retrieved by an extensive literature search and very few datasets were found to support the model development for the BPA alternatives.

To dynamically assess exposure and bioaccumulation, the simplest models are based on a one-compartment assumption, according to which the chemical concentration is the same throughout the organism. This model is suitable for compounds that distribute rapidly throughout the body [38]. This approach has been mainly applied on BPA for various aquatic organisms: in freshwater clam [39], in frogs [40,41]) and in fish [42,43]. To our knowledge, only one publication has explored the bioaccumulation of the bisphenol analogues using a simple one-compartment model [42]. These authors have studied bioconcentration of eight common bisphenol analogues, including bisphenol A, -B, –C, -E, –S, -Z, -AF, and -AP in common carp and calculated bioconcentration factors based on the total bisphenols. These authors suggested that kidney and liver played important roles in accumulating bisphenols in carp, and kidney made more contribution than liver for most bisphenols. In addition, they concluded that biliary excretion predominated for elimination of most bisphenols while BPA and BPS were excreted mainly through urine [42].

In fish, several PBPK models have been used to predict the BPA bioaccumulation [44–46]. However, only [46,47] are specific to this compound and explore its ADME process. These two models were developed based on the experimental data already published and have also generated new data. [46] developed a PBPK model to explore ADME of the BPA and its main metabolites: BPA-monoglucuronide and BPA-monosulfate. These authors conclude that the BPA ADME process was similar between stickleback, zebrafish, and trout and that plasma or gills could be a non-negligible site of BPA metabolization. [47] explored also the toxicokinetic of bisphenol alternatives. These authors present a new dataset on the distribution of BPZ in female zebrafish, measured *in vitro* liver metabolism for 11 alternative bisphenols and adapted a PBPK model for three of these alternative bisphenols: BPZ, BPAF and TBBPA. This work suggests that studied bisphenols mainly distribute to the carcass and gonads and less to the brain. These works based on physiology do not observe a clear role of urinary excretion for the elimination of bisphenol analogues, contrary to the study done by [42].

Toxicodynamic models

Improving the environmental risk assessment of bisphenols requires a robust understanding of ecosystem functioning and population dynamics under toxicant stress, which is one of the challenges of ecological modelling [48,49].

At the organism level, a Dynamic Energy Budget (DEB) model was developed to simulate the effects of BPA on rainbow trout [50]. This modeling study utilized original experimental data to examine various modes of action on DEB parameters. The study indicates an imprinting effect of BPA on energy mobilization from reserves [50].

Regarding higher biological levels such as population, community, and ecosystem, to the best of our knowledge, only two population models have been developed to assess the effects of BPA. These models include a stage-structured Lefkovitch matrix model applied to mosquitoes [51] and an Individual-based model coupled with a DEB model applied to a fish (three-spined stickleback) [49]. The population dynamic model for mosquitoes predicts negligible consequences on the populations under two BPA exposure scenarios (short and long-term) [51]. The fish model was developed to predict the population-level impacts and, additionally, to provide insight on the mechanisms of BPA toxicity from mesocosm data. Modelling results showed that direct BPA effects on fish mainly explained the impacts on the population structure [49].

4.4.4. Conclusions and perspectives

The review on QSAR highlights two major issues: firstly, the insufficient representation of bisphenols in the training sets, along with the scarcity of experimental data for compounds resembling BPs. Secondly, there is a substantial demand for comprehensive *in silico* methods that encompass the entire group of bisphenols, including their derivatives and alternatives. This demand stems from the significance of endpoints, which also hold regulatory importance.

The dynamic models analyzed in the TK part of this review suggest potential variations in ADME processes among bisphenol analogues, which requires additional research to verify. Regarding the TD aspect, this review emphasizes the necessity for predictive models capable of assessing the impacts of bisphenols across various levels of biological organization, from the organism to the ecosystem.

4.5. Biological activity of bisphenols

BPA and its substitutes can be detected in a wide range of materials that can degrade and release bisphenols into the surrounding environment. Consequently, BPA alternatives have been found in diverse environmental compartments, including the atmosphere, water bodies, soil, and sediment, as discussed in Chapter 3. While the biological effects of BPA alternatives have been studied in relation to human health, given their presence in plastics and packaging materials that come into direct contact with humans, it is equally important to delve into the ecological impact of these substances in the environment. This includes a focused investigation of their impact on the environment, particularly on the various forms of life inhabiting it.

In the upcoming chapters, we methodically outline the existing knowledge regarding the biological effects of BPA alternatives through a wide range of model organisms and conducted exposure experiments. This synthesis aims to provide a thorough comprehension of the influence of BPA alternatives on various organisms, spanning from microbiomes to vertebrates, in order to assess the toxicity of these compounds and pinpoint areas where further research is needed.

4.5.1. Microbial organisms as single species or microbiomes

Microbial communities play a tremendous role in natural (*e.g.* freshwater ecosystems, soil) and host-associated ecosystems (holobiont). For instance, phototrophic organisms play a critical role in biogeochemical cycles and primary production. While microbial-based bioassays (*i.e.* as monoculture) have been included in regulatory hazard assessment already for a long time with standardized guidelines at ISO and OECD [e.g. ISO 8692:2012 , OECD 201 [52]], the last decade has seen the expanse of microbial ecotoxicology studies investigating the response of free-living or host-associated microbial communities as a whole *(i.e.* microbiome) [53–55]. Altogether, these studies could provide better insight into the potential impairment of ecosystem function and services by chemical contaminants. In this section, we specifically reviewed the effect of BPA and their derivatives on microbes as single species or more free-living complex assemblages (*i.e.*, soil and sediment microbiomes).

<u>Effect on unicellular organisms</u>

The broadest literature about the effect of BPA on single species microbes is on microalgae in both freshwater and marine ecosystems. Indeed, as recently reviewed [56], many studies have shown the ability of BPA to impair the growth, photosynthesis, membrane integrity, and cellular shape of these organisms, and lead to oxidative stress. Fortunately, only few of them have reported such an impact at environmentally relevant European concentration (<10 µg/L). For instance, Chae et al. [57] highlighted that 7 days exposure of *Chlorophyceae* species to BPA in the ng/L range (from expanded polystyrene leachate) triggers an increase of the photosynthetic yield leading to growth stimulation. Also, Rabet at al. [58] reported a decrease of chlorophyll a and cell density and of photosynthetic activity (quantum yield) in the marine dinoflagellate *Alexandrium pacificum* following 7 days exposure to 2 and/or 20 µg/L of BPA, respectively. Interestingly, the same authors did not report any effect of BPA on the diatom *Chaetoceros decipiens* following similar exposure regimes, highlighting that single species microalgal assays might not be sufficient to uncover toxicity effects. A potential explanation for this species' lack of sensitivity could relate to microalgae specific bacterial microbiome which might reduce BPA toxicity, *e.g.* through metabolic degradation [59].

Literature on the effect of BPA derivatives on microorganisms remains very scarce. Czarny-Krzymińska et al. [60] recently investigated the toxicity of the BPAF, BPG, BPM, BPX, BPA, BPY, BPP on the green algae species, *Chlorella vulgaris* and *Desmodesmus armatus*. Here, effects were observed at quite high concentration (5-100 mg/L) and demonstrated that BPA substitutes were more actively inhibiting growth than BPA (*i.e.*, BPG>BPX>mixture>BPAF>BPA>BPY>BPP) and that the combined exposure to BPA and its structural congeners led to synergistic effect. [61] evaluated the effect of BPA, BPS and their mixture on the green algae *Chlorella pyrenoidosa* following 6 days exposure in the 1-100 mg/L range. At these concentrations, both compounds caused inhibition of growth (cell density) and chlorophyll *a* biosynthesis while inducing oxidative stress as determined by measuring increased levels of ROS, a modulation of MDA (malondialdehyde) content and the induction of SOD (superoxide dismutase) and peroxidases activity. This study also highlighted an overall synergistic effect of the combination of BPA and BPS. Finally, there is also evidence that BPA and its derivatives can have anti-cyanobacterial activity. Indeed, Czarny et al. [62] reported a reduced chlorophyll *a* content and growth in *Anabaena variabilis* and *Microcystis aeruginosa* following a 14 days exposure to BPA, BPAF, BPB, BPBP, BPC, BPE and their mixture in the 1-100 mg/L range. Also, they highlighted that some of the analogues were more active than BPA with a ranking of the effect

magnitude that is species-dependent: BPAF>BPC>BPB>BPA>BPE>BPBP for *Anabaena variabilis and* BPB>BPAF>BPC>BPA>BPE>BPBP for *Microcystis aeruginosa*.

Effect on free-living microbiomes

Beyond the effects on host-associated microbiomes, there is also recent evidence that BPA and its substitutes impair the functioning and biodiversity of environmental microbiomes. Almost 10 years ago, Yang et al. 2014 [63] demonstrated that BPA decreased bacterial community diversity in sediments, likely by promoting the growth of tolerant species capable of degrading the chemical. More recently, Zaborowska et al. 2020 [64] evaluated the response of soil microbiomes to BPA, BPF and BPS at both the functional (i.e., enzymatic activities) and structural (*i.e.* counts and diversity of bacteria) levels. They demonstrated that all tested bisphenols altered both the bacterial composition (*e.g.*, reduction of *Proteobacteria* and increase of *Actinobacteria*) within soil as well as modulated bacterial enzymatic activities (*i.e.*, mainly arylsulphatase and urease) as a function of exposure time and bisphenol diversity. This revealed that BPF was more active towards soil microbiomes than BPS and BPA. Finally, by using metagenomics, Tong et al. 2021 [65] demonstrated similar structural shifts (*i.e.*, decreasing diversity and richness) and associated changes in the functional potential of soil communities following 28 days exposure to BPA. This revealed that the pattern of response, as well as the biodegradation capacity towards BPA, differed according to soil types *(i.e.*, various mangrove rhizosphere soils vs. unplanted soil).

Together, these findings highlight that environmental and host-associated microbiomes are impacted by bisphenols at both the functional and structural level, which might lead to unanticipated impacts on ecosystem functioning and associated services. Thus, we advocate that microbes and their communities should be integrated into standard hazard assessment workflows. To this end, already available protocols could be used to routinely monitor the impact of BPA and its alternatives on the physiology, functioning and structure of microorganisms including host-associated microorganisms [66–68]. Finally, the development of additional cutting-edge assessment methods (e.g. omics, high throughput microphysiology) will help facilitate the rapid testing and monitoring of exposure effects on microorganisms of importance to ecosystems and human health.

4.5.2. Invertebrates

One of the priorities at European level as presented in Chemicals Strategy for Sustainability and other policy documents is to ensure that the endocrine disruptive substances are recognized in a timely manner and that exposure of humans, and the environment is minimized. One of the means to achieve this goal is the development and uptake of methods to generate information on endocrine disruptors through screening and testing of chemicals. In addition, the need for the development of new methods for the identification of possible adverse effects on endocrine system of specific categories of organisms, mainly invertebrates, has been highlighted by EFSA (European Food Safety Authority), ECHA (European Chemicals Agency) and other relevant authorities with the technical support of the Joint Research Centre (JRC). In the Final Report of the State-of-the-Art Assessment of Endocrine Disruptors [69], it is highlighted that biotests with invertebrates offer some advantages over vertebrate models, namely due to the fact that there are fewer ethical considerations, doses are easier to deliver precisely in aquatic species, and their small size and inexpensive maintenance requirements allow larger datasets to be collected. Although some of them have been explored so far, invertebrate models have much to offer in terms of investigating EDC

effects relevant to vertebrates and as ecotoxicological models for screening chemicals for potential ED properties. Many endocrine pathways are evolutionary well-conserved, and despite existing knowledge gaps, in particular for most invertebrate phyla, it is clear that there are structural and functional commonalities across vertebrate and invertebrate taxa. However, the endocrine system of invertebrates is different than for vertebrates [70], which suggests also other targets for endocrine disruption, and well-known vertebrate EDCs may interfere with the development, reproduction and endocrine system of invertebrates via other pathways than the estrogen, androgen, thyroid and steroidogenesis pathways *e.g.* the retinoid-signaling pathway. How vertebrate EDCs affect most invertebrate taxa is largely unknown. Better understanding of the effects of EDCs on invertebrates could not only help better protect invertebrates in the environment, but also permit future extrapolation of knowledge across species and thereby help reduce the need for vertebrate testing. Therefore, in this chapter the current state of knowledge regarding the toxicity of BPA and its alternatives on invertebrates has been reviewed and is presented in Supp.info 5 (Tab.1) with the aim to identify possible gaps in their toxicity assessment with classic (standardized) methods and development of NAMs. Based on the review of more than 70 publications (listed in the Supp.info 5 (Tab.1), our investigation identified that most of the available data still refers mostly to BPA (~80%), and only 16 BPA alternatives have been toxicologically evaluated towards different invertebrate species so far. It might be also concluded (similarly to the data referring to vertebrate models) that BPA alternatives have not been studied systematically. For example, there is a lack of systematic approach in terms of the evaluation of a specific endpoint. The effects of BPA alternatives are also unequally investigated. Three BPA alternatives that were studied most commonly are BPS (22%), BPF (14%), and BPAF (6%).

This highlights the gaps in the ecotoxicity assessment of BPA alternatives. Detailed information is presented in the following sub-sections referring to the specific groups of invertebrates.

Terrestrial and aquatic insects

Insects are the most common animals on earth with more than 80% of animal species being insects. Insects are of high importance to terrestrial and aquatic ecosystems since they are involved in a series of essential processes related to ecosystem maintenance *e.g.* pollination, decomposition and nutrient recycling, soil formation, etc. In addition, insects play a critical role in food webs and as predators provide natural population control of other insects, arthropods and other invertebrates [71]. Therefore, there is a growing concern as regards the global decline in insect biodiversity and the possible adverse effects caused by several parameters (including the environmental chemical pollution) on insects' survival, reproduction and on different systems (*e.g*. endocrine system).

So far, endocrine disruptive effects on invertebrates and specifically on arthropods/insects were only assessed through reproduction studies that do not specifically address ED related endpoints. In parallel, certain chemicals that are emerging environmental contaminants like some BPA alternatives have been shown to exhibit adverse effects on the endocrine and immune system, on reproduction and metabolism of invertebrates, and therefore further investigation is required. As regards the endocrine system, several studies were focused on the effects on ecdysis. Ecdysis is the process of an arthropod moulting its exoskeleton. Moulting is necessary as the arthropod exoskeleton is inflexible and so, to grow larger, arthropods must moult. Therefore, successful moulting/ecdysis is a critical event for the development, survival and reproduction of arthropods. Ecdysis is controlled by complex multi-hormone systems and therefore it can be affected by endocrine disruptive chemicals (EDCs).

More specifically, for example, exposure to environmentally relevant concentrations of BPA during the early stages of the housefly *Musca domestica* life cycle variously affects its development. The observed effects (*i.e.* alteration in pupal weight, sex ratio, and levels of juvenile hormones) were related to endocrine disruption [72]. Several studies indicate that BPA interferes with the ecdysteroidal pathways of the lepidopteran insect species. BPA exhibits ecdysone agonistic activity and causes developmental delay, alterations in pupal and larval weight, decreased adult emergence and malting malformations during development and metamorphosis [73,74]. These effects were correlated with modification of expression of various genes involved in ecdysis process: EcR, USP, E75AB, E75D, Br-c [75], SnEcR and SnUSP [73], SnoHsc70 [74], SnoHsp19.5, SnoHsp20.8, SnoHsp83 [76].

BPA exhibits endocrine disruptive effects also on *Drosophila melanogaster* by affecting endocrine signaling through disrupting hormone levels and/or gene expression levels of hormone receptor or signaling pathways controlled by these hormones [77,78]. Besides the ED related effects, BPA induces gastrointestinal toxicity in *D. melanogaster* [79] and was involved in the regulation of metabolic pathways, behavioral patterns, stress response, endocrine homeostasis, neural functioning, and the development of specific organs in *Drosophila* [80]. In *Culex quinquefasciatus,* BPA treatment caused decrease of the time for embryonic and larval development. More specifically, BPA caused a dose-dependent increase of 20-hydroxyecdysone (20-E) peaks, phospholipase A2 induction and upregulation of ecdysone receptor gene, EcRA, and ecdysone inducible gene E75A, which results in early pupation [81].

BPA and other bisphenols also affect aquatic insects. More specifically, exposure to sublethal concentrations of BPA causes delayed moulting and decreased larval wet weight in *Chironomus riparius* [82]. In addition, BPP alters embryo hatching, larval emergence, and adult sex ratio at concentrations close to the effective concentrations for hormonal genetic endpoints in embryos and larvae after 48 h of exposure [83]. BPA and other bisphenols also affect the expression of genes involved in the endocrine and other systems of aquatic insects. In *C. riparius*, BPS causes alteration in several genes related to the ecdysone pathway and metabolism (EcR, ERR, E74, cyp18a1, swadow) and other genes crucial for insect development and metamorphosis, stress and biotransformation mechanism (hsp70, hsp40, cyp4g, GPx and GST) [84,85]. Other studies indicate that EcR and E93 were statistically significantly upregulated in response to BPA, while in parallel reduced transcription of JHAMT and DECAY genes was observed [86].

Besides the endocrine system, BPA was found to have genotoxic activity on *C. riparius* as demonstrated by tests other than basic ecotoxicity assays, *i.e.* comet investigating DNA damage eco-epigenetics (global DNA and histone methylations) and non-targeted global metabolomics (NMR based) approaches [87,88].

In conclusion, there are several indications that BPA and BPA alternatives influence the endocrine system of insects through affecting the ecdysis pathway. Therefore, these results highlight the need for the development of methods to include not only morphological assessment but also molecular, analytical and omics techniques to identify molecular markers for the assessment of the possible adverse effects on an insect's endocrine system. These molecular markers can also be used to enrich the existing adverse outcome pathways (AOPs) or/and to develop new AOPs related to the ecdysis process in invertebrates.

Mollusks

Mollusca is the second-largest invertebrate phylum consisting of >85,000 species, whereof 80% are gastropods. Combined with their commercial and ecological importance and their use as biomonitoring organisms, this phylum has gained particular research focus among invertebrates. Mollusks could offer

predictive capabilities for understanding effects of chemicals in vertebrates as they have closer evolutionary relationships with vertebrates than other protostome invertebrates such as the arthropods [89].

Two mollusk species, both being freshwater gastropods, are used as model species in OECD test guidelines (TG); the New Zealand mud snail (*Potamopyrgus antipodarum)* in TG 242 and the great pond snail (*Lymnaea stagnalis) in* TG 243. Both TGs were adopted in 2016 and are 28-days toxicity and reproduction tests with sexually mature snails, but without endocrine specific endpoints. Therefore, major gaps remain in understanding the role and function of the endocrine system in e.g., development and reproduction in mollusks and how pollutants interfere with endocrine pathways. Thus, the mechanisms of endocrine disruption and the relationships to hormonal changes and reproduction disturbances are not fully clear yet. Vertebrate nuclear receptor (NR) orthologues such as ER and TR have been identified in mollusks including gastropods. However, evolutionary changes in binding specificities and activation profiles of NRs are well-known as structure is better conserved than function. For example, accumulating evidence suggests that the mollusk estrogen receptor (ER) is unable to bind vertebrate-type estrogen (E2) and has its function as a constitutively active transcription receptor [90]. Hence, the actual functionality of the molluscan ER *in vivo* still needs clarification. Although mollusks can capture, store, and metabolize vertebrate-type steroid hormones, there is no conclusive evidence that they can synthesize vertebrate-type steroid hormones endogenously [91], and in contrast to fish, yolk protein is not induced by vertebrate E2 or EE2 in bivalves [92,93]. It has been suggested that estrogens could act through non-genomic signaling pathways in bivalves [94].

Due to a fairly well-characterized reproduction, development, and nervous system, *L. stagnalis* is both a well-established multipurpose model within neuroscience and evolutionary biology, and a promising mollusk model organism in ecotoxicology [95]. *L. stagnalis* is a freshwater hermaphroditic snail commonly found in Europe and North America [95] and it is easily maintained under laboratory conditions. The snails are hermaphrodites that reach sexual maturity a few months after hatch and can produce high numbers of offspring all year round. The embryonic development is easily studied due to large and transparent individual eggs within a transparent egg cocoon. The species is thus well suited for assessing developmental and reproductive toxicity of chemicals. There is a need for standardized assessment of chemical toxicity, especially for early life stages such as embryos as they are often more sensitive, which is also the case for BPA. Therefore, *L. stagnalis* (adults and embryos) is used for NAM development within PARC. However, some biological areas need further research to fully understand and utilize the full potential of *L. stagnalis*. The genome of *L. stagnalis* has not yet been fully characterized, and there is a need for further genome identification which can allow for knowledge and development of molecular toxicity biomarkers. Consistently, there are very few studies using “omics” techniques for assessment of chemical toxicity in *L. stagnalis*. In addition, other biomarkers reflecting the organism health, such as biochemical, histological, morphological, behavioral, and physiological measures, should be further explored as well.

BPA and BPA alternatives have been detected in mollusks in the environment in the ng/g tissue range [96]. The literature concerning the effects of BPA on mollusks was comprehensively analyzed in the support document for identification of BPA as a Substance of Very High Concern (SVHC) based on endocrine disruptive properties [97]. Embryo malformations, increased egg production, induction of super females and malformations of genital tissue were among the main findings. Effect concentrations were in the low µg/L range (high variation in sensitivity between species, studies and laboratories) and embryos being more sensitive than adults, but the precise mechanism is not clarified yet. The effects could be highly dependent on life stages, exposure window, and specific concentrations at specific time points which is

already well-documented for the endocrine disruptive effects of tributyltin (TBT) on marine mollusks. *Marisa cornuarietis* and *Potamopyrgus antipodarum*, two prosobranch snails, were the most extensively studied. Since 2017, only a few *in viv*o single exposure BPA studies in mollusks have been published. In the gastropod *Pomacea lineata*, Andrade *et al.* (2017) [98] found decreased neonate heart rate, adult behavioral changes and 96-h LC_{50} values of 3.14 mg/L (neonate) and 11.1 mg/L (adults) which is comparable with earlier gastropod studies [99]. In bivalves, BPA exposure caused impaired antimicrobial ability (Tang et al., 2022) and female-biased sex ratio [100].

The literature concerning toxic or endocrine disruptive effects of BPA alternatives in mollusk *in vivo* studies is limited to a few reports on the effects of TBBPA. The effects of TBBPA were investigated and compared between several vertebrate and invertebrate species including two bivalve mollusks: the Eastern oyster (*Crassostrea virginica*) and the blue mussel (*Mytilus edulis*). Mollusca was the most sensitive aquatic phylum and shell growth was the most sensitive endpoint in both short-term (96-h, *C. virginica*) and long-term (70-d, *M. edulis*) exposures with NOEC and LOEC values in *M. edulis* of 0.017 and 0.032 mg/L, respectively (reviewed by Pittinger & Pecquet, 2018 [101]).

The fact that BPA and BPA alternatives have been detected in mollusks in the environment [96] shows the importance of including mollusks in further studies of BPA and its alternatives. Especially early life stages of mollusks seem to be sensitive towards BPA exposure, but the effects of BPA alternatives, except TBBPA, are not investigated in mollusks. *L. stagnalis* (adults and embryos) as a model species appears to be appropriate due to the promising toxicity testing methods currently under development. In *L. stagnalis* (adults and embryos) neither BPA nor its alternatives have been tested and an evaluation of the impact of BPA and its alternatives in *L. stagnalis* (adults and embryos) should be initiated by studies of BPA itself and followed by single and combined exposures with BPA alternative.

Ascidian tunicates

The vase tunicate (*Ciona intestinalis*), which is an invasive ascidian tunicate, has been important model organisms in developmental biology and genomic evolution research for more than a century, due to their phylogenetic position as the earliest chordates, and the potential for using ascidian tunicates as ED bioindicators has gained increasing attention lately [102–104], and the topic was recently discussed [105]. The increasing interest on *Ciona* (and related ascidians) is also due to their wide distribution and abundant presence in shallow coastal seas, uncomplicated maintenance in laboratory cultures, rapid embryo development and short life cycle, well characterized developmental ontology, tadpole-like swimming larvae, the transparent body of the sessile adults, and their fully sequenced genome. With tunicates being the closest living invertebrate relatives to all vertebrates, the effects caused by EDCs on these test models could hypothetically have broad relevance, maybe in some cases for both invertebrate and chordate taxa. However, the degree by which Ciona/ascidians are usable as bioindicators in ED assessments relies on the amount of knowledge that is available on their endocrinology, their sensitivity and responsivity to known EDCs, as well as the degree to which their putative ED responses are comparable to other taxonomic groups. These are topics for which there are still many knowledge gaps. But notwithstanding these challenges, using ascidians as test species for assessing ED properties of BPA and its substitutes could be an option worth considering, and this constitutes the scope of this chapter.

About 40 types of hormones/neuropeptides have been identified and characterized in ascidians by purification, cDNA cloning, and peptidomic approaches, and the main ascidian hormones/peptides that have been found can be classified into three categories: (1) vertebrate homologs, such as gonadotropin-

releasing hormones, tachykinins, galanin-like peptides, calcitonin and insulin/relaxin paralogs; (2) hormones/peptides that belong to conserved families but with distinct sequences or activities compared to vertebrate homologs, such as GnRH-X and vasopressin; and (3) hormones/peptides that are ascidian-specific, such as Ci-YFV/Ls (novel neuropeptides specific to *Ciona* with unknown biological functions but which share a Tyr-Phe-Val/Leu sequence at the C-terminus) and Ci-LFs (eight novel neuropeptides possibly specific to *Ciona* that share a Lys-Phe sequence at the C-terminus), see multiple references in Beyer et al. 2023 [105]. However, the function of many of these hormones/peptides remain uncharacterized. Nuclear Receptors (NRs) that are positively identified in ascidians could represent important focus points for studies into possible ED effect phenomena and ED related MOAs in ascidian taxa. Studies of the sequenced genome of *Ciona* have identified at least 17 genes which encode for NR transcription factors [106]. NR genes documented to be present in ascidians include, among others, the thyroid receptor (TR), the peroxisome proliferator-activated receptor (PPAR), the retinoic acid/retinoid X receptors (RAR/RXR), the vitamin D/pregnane X receptors (VDR/PXR), and the estrogen-related receptor (ERR), whereas NR related genes that are present in humans, but thought NOT to be present in ascidians, include most importantly the steroid hormones and their receptors, i.e., the estrogen receptor (ER), androgen receptor (AR), mineralocorticoid receptor (MR), glucocorticoid receptor (GR), and progesterone receptor (PR), for references to original studies see Beyer et al. 2023 [105].

BPA is possibly the ED substance (beside tributyltin, TBT) which so far have been most studied in ascidians, although the total number of studies is still quite limited, i.e.,[107–116].

To highlight some of these studies, Messinetti et al. (2018; 2019) [107,108] investigated dose-responsive effects of BPA on ontogenetic development in larvae of two ascidian species, *Ciona* and the related ascidian *Phallusia mammillata*, identifying specific disorders (in neural differentiation and swimming tail and pigmented organs morphology) as markers of ED effects, and suggested also which NR system (Estrogen-related receptor, ERR) and which target cells (GABAergic and dopaminergic neurons) that were affected by the BPA exposure. Involvement of upregulation of ERR activity was suggested as MOA as co-exposure of ascidians with 4-hydroxytamoxifen, a compound that binds and deactivates ERRs, resulted in normal pigmented-organ phenotypes. Gomes et al. (2019) [102] addressed the role of ERR in neurodevelopmental toxicity of bisphenol A to embryos of the ascidian *Phallusia mammillata*. Cangialosi et al., (2013) [109] assessed the effect of BPA and atrazine in *Ciona* embryos and found concentration-dependent effects on embryo development (development arrest, fused or absent blastomeres and lack of follicular and test cells). Matsushima et al. (2013) [110], investigated how low (1 microM) BPA exposure of early (neurula stage) embryos adversely affected the swimming behavior of the subsequent larva. The study suggested a mechanism that involved BPA binding to and disrupting the regulatory action of ERR receptors, although the molecular mechanism was not clarified. Mansueto et al. (2011) [111]used post-larvae juvenile *Ciona* as test models for detecting impacts of BPA (and TBT) on organ morphology developments and found dose-responsive effects on the morphology of tunic, gonad cells, nervous system, digestive system, as well as and inhibition effect of the rhythmic body contractions. Mercurio et al. (2022) [112]studied teratogenic effects of BPA and the flame retardant tris(chloro-propyl) phosphate on embryo development in *Ciona*. Lastly, Richter and Fidler (2015) [113] explored the potential of reporter gene bioassays developments based on ascidian NR genes (ascidian VDR/PXRα LBDs as fusion proteins combined with GAL4-DBD, a generic transcription activation domain (VP16-AD) and the lacZ reporter gene) as high-throughput test tools for emerging substances with ED mechanisms similar to BPA.

Ciona intestinalis is considered a promising model test species for developing new approach methodologies (NAMs) for ED related testing and screening, both regarding BPA substitutes and other chemicals of emerging concern. As tunicates are the most primitive chordates alive, such NAMs will meet

the requirements on chordate test animal ethics. However, considerably more research/knowledge is required, both on the endocrine system in *Ciona*/ascidians and how it will be interfered with by different EDC classes, before the usefulness of *Ciona* as an ED model test species can be assessed. Although many vertebrate hormone receptor paralogs have been identified in tunicates, their ligand-binding status is still largely unknown. Hence, it will still be premature to conclude that *Ciona* can serve as an alternative test model for screening BPA substitutes and other, possibly ED acting, emerging contaminants.

Crustacea

D. magna is a small freshwater crustacean that plays a crucial role in the food chain and in the freshwater ecosystem, as it consumes mainly green algae and simultaneously is the main food source for fish. Therefore, its reproduction or behavioral disorders may influence the balance of the aquatic ecosystem. Considering its common distribution, short life cycle, large reproduction capacity as well as stable genotype and sensitivity to different chemicals *D. magna* is used as a model organism in aquatic toxicity tests of different environmental pollutants [117]. The influence of specific compounds on *D. magna* can be assessed based on the different behavioral and physiological responses, such as *e. g.* immobilization, mortality, moulting, feeding, swimming and reproduction [118] . In general, the reproduction in culture is leading to the production of females by diploid parthenogenesis. Males are only produced in response to environmental stress (*e.g.* high population densities). The body of a *Daphnia* is not clearly segmented, with the thoracic and abdominal regions covered in a secreted shell or carapace [119].

Brennan et al. (2006) [119] highlighted that in crustaceans many physiological processes are regulated by neurohormones, which includes lipid metabolism, digestion, ionic balance, moulting, growth, regeneration, reproductive physiology, gonadal development as well as cardiac activity [120]. Moreover, most of the invertebrate hormones have been identified as peptide neurohormones, however there has not been enough evidence on the interference of EDCs on peptide-base signaling systems. However, it has been also highlighted that key insect and crustacean hormones are non-peptide based, such as juvenile hormones or ecdysteriods, and processes in which these hormones are involved may be more susceptible to receptor related interference. For example, it has been hypothesized that some chemicals which disrupt endocrine processes in vertebrates can also negatively affect the hormonally related moulting process in arthropods by acting as antagonists of endogenous ecdysteroids resulting in the blocking [121] or activation of the ecdysteroid receptor [122] . The potential of chemically induced molting interference has been the subject of several putative (conceptual) AOPs involving chitin synthesis and degradation (AOP #358, #359 and #361) and as well as one OECD endorsed AOP (AOP #360) collectively organized under the OECD project 1.94 (www.aopwiki.org). Other AOPs of relevance include ecdysone receptor-mediated interference with molting (AOP #4) and interference with male sexual development (AOP #201). Increasing attention is given these toxicity mechanisms [122–126] in crustaceans such as *D. magna*, although the number of chemicals tested remains low. Nevertheless, the assessment of interference within the endocrine system of invertebrates is difficult to predict. Despite these mentioned difficulties with interpretation of the *Daphnia* tests' results for risk assessment strategy of EDCs [119], *D. magna* seems to be a valuable model for evaluating endocrine disruptors' effects.

D. magna was also used for the acute and chronic toxicity assessment of BPA and its alternatives Supp.info 5 (Tab.2). The available data for acute toxicity (including mainly standard endpoints such as immobilization or mortality) is also presented in the Supp.info 5 (Tab.1). It might be concluded that most of the available data refers to BPA and the information about the toxicity of BPA alternatives is very

inconsistent and limited. Even less data is available for the assessment of long-term effects of BPA alternatives towards D. magna (Supp.info 5 - Tab 1).

With respect to the prolonged exposure of *D. manga* to BPA and its alternatives, many different endpoints were investigated. This specifically includes not only reproductive indicators (such as: time to first brood, number of first brood per female, brood times, total number of offspring, number of offspring per brood per female and intrinsic rate of natural increase) as well as growth and development parameters (including swimming behavior, body length, heart rate and thoracic limb activity), but also biochemical indicators (acetyl choline esterase (AChE), superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) activities as well as malonic dialdehyde (MDA) content) [127].

Based on the available results it was confirmed that relative activity of these enzymes in *D. magna* decreased after exposure to BPA, BPF, BPS and their mixtures, implying that they may cause the disorder of digestive, nervous and antioxidative system of *D. magna* [118]. Similar observation were also made in the case of BPF, which induced the disturbance of neurological function and oxidative stress situation in *D. magna*, leading to behavioral and reproductive effects, such as growth inhibition, delay in sexual maturation, decreased spawning, and eventually reduced population fertility [127]. Moreover, the observed correlations between biochemical, behavioral, and reproductive endpoints indicated that the potential of biomarker techniques as early predictors of bisphenol contaminants-induced alterations is also at a higher sensitivity level.

The available data presented in this chapter highlight the fact that more comprehensive studies on the adverse effects of BPA and its alternatives on aquatic organisms are required at different levels of the ecological hierarchical scale [127]. Several marine crustaceans have been proposed as potential candidates to investigate the biological effects of contaminants on primary consumers. Brine shrimp *Artemia* spp. (Crustacea, Branchiopoda, Anostraca), commonly known as sea monkey, is a cosmopolitan organism inhabiting saline environments, such as inland saltwater lakes and coastal lagoons, feeding primarily on phytoplankton and being an important primary consumer. The main advantages of brine shrimp which make it a good test organism is the short life cycle thus a short test duration (i.e. 28–72 hours from hatching to the first endpoint) and the easy culturing, maintenance and manipulation under laboratory conditions. Moreover, the use of nauplii hatched from commercially available durable cysts (eggs) ensures population homogeneity (organisms of similar age, genotype and physiological condition) and availability all year-round without the necessity of culturing [128].

Artemia's sensitivity, like in most aquatic organisms, depends on its developmental stage. According to literature, instar II-III (larval stage) have been shown to be the most sensitive stage [129,130]. One of the most important variables in the performance of any biological experiment is the nutrition of the test animals [131]. In this context, one of the important advantages of *Artemia* is that nauplii (stage II-III) do not require feeding for up to 48 hours. Therefore, in assessing the ecotoxicity of pollutants, the acute toxicity test with mortality / immobilization (after 5–30 s observation) as the end point is most often used. Besides, sublethal indicators of toxicity have already been applied for *Artemia* as biological endpoints, such as biomarkers (acetylcholinesterase; heat shock proteins; lipid peroxidation; enzymatic biomarkers of oxidative stress such as glutathione peroxidase; glutathione S-transferase; glutathione reductase), hatching (dry biomass, morphological disorders and size), behavioral changes (swimming speed and path length), and genotoxicity. Long-term chronic tests focus on growth, reproduction and survival or mortality after 7–28 d exposure from larval to adult stage [132].

Very good interspecies correlation between *A. salina* and other important aquatic organisms used for ecotoxicological testing has been observed. It was concluded that *A. salina* could be used as an

572 appreciable tool for prediction of toxicity to other organisms, especially to fish [133]. *Artemia* spp. was
573 also used for the acute toxicity assessment of BPA (Tab.5). Unfortunately, ecotoxicity data for BPA
574 alternatives obtained using this species are not available so far.

575 **Table 5.** The acute toxicity of BPA to Artemia species.

Species **life stage**	**Endpoint**	**LC_{50} [mg/L]**	**Other observations/conclusions**	**Reference**
Artemia franciscana nauplii (instar II-III)	mortality	$LC_{50,\ 24h}$ = 44.8; $LC_{50,\ 48\ h}$ = 34.7	BPA exposure influenced nauplii growth; dose-length relationship was observed	(Castritsi-Catharios et al., 2013) [134]
Artemia franciscana nauplii (instar II-III)	mortality	$LC_{50,24h}$ = 45.51; $LC_{50,\ 48h}$ = 34.45, $LC_{50,72h}$ = 17.12	growth inhibition was observed and was suggested as a valid endpoint for toxicity studies	(Ekonomou et al., 2019) [135]
Artemia salina adult	mortality	$LC_{50,96h}$ = 107.2	BPA toxicity increased towards higher trophic levels (microalga (Tetraselmis sp.)< zooplanktonic grazer (*A. salina*)< deposit-feeder invertebrate (*Heleobia australis*)< omnivorous fish (*Poecilia vivipara*)	(Naveira et al., 2021) [136]
Artemia salina naupli (instar II-III)	immobilization	$EC_{50,\ 24h}$ = 56.1		(Kalčíková et al., 2012) [133]
Artemia sinica	mortality	$LC_{50,\ 24h}$ = 70.1;		(Shaukat et al., 2014) [137]

		$LC_{50,\ 48h}$ = 50.4; $LC_{50,\ 72h}$ = 17.3		
Artemia sp. nauplii (instar II)	mortality	$LC_{50,\ 24\ h}$ = 74.7; $LC_{50,\ 48h}$ = 59.4	PNEC (24h) = 74.6; (48) = 59.3; RQ (24h) = 1.02×10^{-6} (48h) = 1.29×10^{-6}	(da Silva and de Souza Abessa, 2019) [138]

576

The results of a study of influence of brominated bisphenol TBBPA on survival (LC_{50}) of the brine shrimp *A. salina* indicated that brine shrimp is more susceptible to TBBPA toxicity in comparison to other aquatic organisms such as microalgae, crustaceans (*D. magna*, copepods) and fish. The study revealed that *Artemia* can also be used in tests based on biomarkers, such as enzymatic activity. The impact of sublethal concentrations (LC_{25}) of TBPA on the activity of enzymes involved in the cellular antioxidant system (GST and GPx), energy metabolism (LDH) and neurotoxicity marker (AChE) was also observed [139].

In all studies listed in Supp.info 5 (Table 1), time and concentration-dependent effect of BPA on mortality was observed. The other effect of BPA which is inhibition of *Artemia* nauplii growth can be estimated within a short exposure period (24 h), even at doses lower than the median lethal concentration [134]. It was suggested that mechanisms of BPA action in *Artemia* may be related with its endocrine disrupting activity. This is in line with the effects summarised in insects' section, since BPA has been demonstrated as a weak ecdysteroid antagonist in insects [140] and in daphnids [141]. In arthropods, including crustaceans, ecdysteroid hormones participate as endocrine-signaling molecules and control biological functions like molting, growth, reproduction and embryogenesis [125,142,143].

Nematoda

Caenorhabditis elegans is a well-established model organism with many favorable characteristics for studying toxic effects at different developmental stages and across several generations. *C. elegans* are relatively easy and cheap to maintain, have a short life cycle of approximately four days (at 20°C) and are highly reproductive. These are prime assets for reproductive and transgenerational studies and thus, the assessment of endocrine disruptive effects [144]. *C. elegans* is a hermaphroditic nematode species with bilobed gonads which, under certain circumstances (like stress or toxic exposure), develop a small minority (~0.2%) of XO males by non-disjunction of the X chromosome [145], which produce sperm and mate with the hermaphrodites. The transparency of the worms allows for easy insight into the germ cell development and the processes during meiotic differentiation, spermiation, ovulation and fertilization. This offers the opportunity to study these processes under chemical exposure to identify germ cell toxicity. Also, the manifold of available mutant and fluorescent reporter strains of *C. elegans* facilitates mechanistic studies *in-vivo* in the live worms.

In terms of BPA and its alternatives, many studies have already demonstrated that *C. elegans* is a suitable model to study the adverse effects of these compounds, from reproductive toxicity to neurotoxicity, which are known or suspected from mammalian and non-mammalian vertebrate studies. There is good evidence that findings obtained from *C. elegans* studies translate into effects in higher organisms [146,147]. The less complex biology combined with the high level of genetic and functional conservation and characterization certainly favor the development of *C. elegans* based NAMs. Invertebrate whole organism models like *C. elegans* are legally unprotected animals (in Europe, except for decapodes and cephalopods) and therefore represent animal-alternatives, and they fulfill the requirements for medium to high-throughput approaches (reviewed by Cornaglia et al. 2017). Moreover, *C. elegans* is equally applicable in the field of toxicology as well as ecotoxicology. Particularly attractive for the ecotoxicology field is *C. elegans*' amenability to artificial and diverse experimental environments and conditions. Toxicity testing can be performed with different media, from agar to soil, sediment and to aqueous media and petri dishes work equally well to microwell-plates. Above all, *C. elegans* represents the ecologically important and biologically diverse organism group of nematodes, which inhabit and often dominate most terrestrial and aquatic habitats (reviewed in Yeates et al. 2009, [148]). Nematodes can thus act as important indicators of ecosystem health [149].

However, there are some limitations that have to be taken into consideration when using *C. elegans* for toxicological studies. *C. elegans* are unable to synthesise cholesterol and therefore require food sources containing sufficient cholesterol or supplementation. Depletion leads to impairment of development, molting, growth and reproduction [150,151]. BPA and structurally similar BPA-analogues like BPS, impact steroidogenesis and it has been shown that this effect is mediated by the disruption of the cholesterol transport between the outer and the inner mitochondrial membrane [152]. It has been shown that cholesterol supplementation under experimental laboratory conditions has the ability to attenuate reproductive effects in *C. elegans* [152–154]. The majority of *C. elegans* studies in the toxicological context add *E. coli* as food supply to their test system. For toxicological examinations, the introduction of another organism with metabolic capacities represents a substantial confounding factor in an exposure scenario [145]. The associated issue of xenobiotic metabolism is not the only aspect; the capacity of the bacteria to act as vehicles for the toxicants as well as recipient adds another level of complexity to the exposure and the toxicokinetics.

Among bisphenols, BPA is probably the best studied compound in *C. elegans*, followed by BPS, BPF, BPAP, TBBPA; TBBPF and TCBPA. The number of publications for each of these compounds according to our research at the time of this publication, was > 20; one publication explored the effects of BPY and BPZ in *C. elegans* [155]. The most commonly assessed endpoints in all of these studies comprised growth, reproduction, behaviors (locomotion, body bends, head thrashes, mechano-sensory habituation, pharynx pumping rate or defecation), DNA-damage (e.g., measurement of DNA damage checkpoint kinase CHK-1 activation) , germ cell apoptosis (e.g., acridine orange staining), stress-related gene expression (e.g. of *hsp-3*, *hsp-4*, *hsp-16.1*, *16.2*, and *hsp-70*) oxidative stress (detection of ROS and antioxidative enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), at protein and mRNA-level) [156,157]. All above mentioned endpoints were found to be affected by exposure to BPA but at varying effect threshold levels between 0.1 and 1.0 µM.

BPA was also able to induce the expression of the vitellogenin gene *vit-1* in *C. elegans* in a concentration-dependent manner [158]. The gene *vit-1* is expressed in the intestine of the worms and the transcription factor *mab-3* (male-abnormal gene) represses vit-1 in male *C. elegans*. The study provides evidence that *mab-3* is repressed by BPA and that this may be linked to the increase in *vit-1* expression.

Epidemiological as well as animal studies have shown that BPA and BPS can have obesogenic effects through a variety of mechanisms like disruption of glucose homeostasis and lipogenesis, inflammation, and oxidative stress [159–161] was able to reproduce these obesogenic effects in *C. elegans*, demonstrating that exposure to BPS increases fat accumulation in the nematodes and that this effect is most likely mediated by an upregulation of fatty acids biosynthesis (via upregulation of the gene *daf-16* in the insulin signaling pathway of *C. elegans*) and inhibition of fatty acid oxidation. Downregulation of the nuclear receptor gene *nhr-49*, which has been identified as one of the key regulators of fat metabolism in *C. elegans* [162], appears to play a central role in the BPS elicited obesogenic effects [160,163]. Similarly, a concentration-dependent increase in lipid deposition was also found in worms exposed to BPA [157].

Lipophilic hormone signaling between the reproductive system and the intestine of the worms can influence adult longevity (Berman and Kenyon, 2006) via the induction of the gene *kri-1* which signals the nuclear translocation and activation of the DAF-16 transcription factor. However, this only happens in germline deficient worms, hence indicating that the aging process is related to the maturation and proliferation of germ cells. Bisphenol compounds have shown to induce oxidative stress in germline cells of *C. elegans* and that this can lead to DNA-damage and subsequently to germ cell apoptosis and

chromosome abnormalities [164,165]. These effects of bisphenols do not only manifest as reproductive toxicity (but also promotes degenerative age-related responses, accelerating the aging process in *C. elegans* [156].

C. elegans appears as a promising organism for the development of NAMs to be used in the context of an invertebrate EDC testing framework. Moreover, chemical screening approaches with *C. elegans* in combination with computational models, hold great potential for the identification of MIEs and KE and thus, the development of invertebrate-specific AOPs.

Annelida (earthworms)

Earthworms are one of the most widely used soil organisms in ecotoxicological studies, as they are excellent bioindicators of contamination [166] and have different standardized tests for toxicity assessment (OECD, 2016, 1984) [167,168]. These organisms are vital for maintaining food chains [169], so their ability to incorporate and accumulate soil contaminants orally or by passive diffusion through the skin can ultimately negatively affect populations of other species. In addition, earthworms may affect soil degradation and the transformation of organic pollutants [170]. Their hermaphroditism makes them excellent ecological candidates for assessing the effects of endocrine disruptors on the male and female reproductive systems in the same organism [171].

Several annelid species have been used to assess the toxic effects of BPA or its alternatives. Since the sensitivity of earthworms to the same chemicals differs even between closely related species, it is helpful to consider results obtained with different species for a more robust toxicological assessment [166].

Two earthworm species (*Dendrobaena veneta* and *Eisenia fetida*) were tested against BPS and compared to BPA toxicity aiming at suggesting it as a potential less toxic alternative [166]. The two species showed different patterns of response towards BPS at 21 ºC. In the case of *E. fetida,* no mortality was observed up to the highest concentration tested (1000 mg/kg artificial soil), with growth being stimulated in the first 14 days but then just showing a trend of decrease in weight similar to the control. In the reproduction test, the number of juveniles was lower at the highest concentration than in the control. In the case of *D. veneta*, no effects were observed regarding their survival, growth or reproduction. In the same study similar experiments were also performed with *D. veneta* at 26 ºC, and some changes were observed, with 10 mg BPS/kg inducing a weight increase higher than the control in the first 14 days of exposure. However, this pattern changed in the last 14 days of exposure, with weight gain observed at 100 mg BPS/kg, while the lowest (10 mg BPS/kg) and the highest (1000 mg BPS/kg) concentrations tested showed earthworms decrease in weight. In a contact test, where only dermal exposure was tested, BPS induced mortality after 48h at 2 mg/ml to these two earthworm species. In addition, earthworms showed oxidative stress responses at 21 ºC, with GST activity decreasing for *E. fetida*while increasing for *D. veneta*. In a separate study [172], these two species were subjected to a contact test, and reproduction traits were only significantly different for E*. fetida*, for which the number of juveniles decreased at higher concentrations (1000-2000 mg/kg), thus showing different sensitivity in both species.

Conversely, another study with BPA and BPS, this time using *Lumbriculus variegatus*, showed that the effects of both chemicals were nearly indistinguishable after 5 min and 5-day exposures. Both chemicals retarded the initial phase of body regrowth after cutting/fragmentation and increased the pulse rate of the dorsal blood vessel (DBV) at concentrations of 10^{-9} or 10^{-6} M [173].

Also, in *L. variegatus*, 1.36 mg/L BPA in the overlying water (57 mg/kg dry weight in sediment) significantly induced biomass loss and a decline in the number of individuals after 28 days of exposure [174]. BPA concentrations above 130 mg/kg significantly induced the death of *E. fetida* individuals in 7-day and 14-day exposures [175], but mortality in this species was also detected in acute 24-h and 48-h exposures to BPA concentrations above 2.42 µg/L [176].

The exceptional sensitivity of earthworm species to some estrogens makes it a possible tool for estrogenic EDC screening. In this regard, a test with *L. variegatus* exposed to BPA and BPS demonstrated that both compounds had a similar effect leading to a significant increase in the pulse rate of the DBV at exposures of up to 10 days in the 10^{-10} to 10^{-8} M range [177]. BPA has been shown to induce endocrine disruption related to the male reproductive organs of *E. fetida* [169], with changes in gene expression of genes EcR, MAPR, AdipoR, along with oxidative stress and multiple histopathological changes in the body wall and ovaries. In a 7-day test with *Eisenia andrei*, BPA inhibited normal oogenesis and maturation of oocytes in earthworm ovary and was observed to damage seminal vesicle tissues and inhibit normal spermatogenesis [171].

Behavioral changes have also been detected in *E. fetida* at the individual level, with an increase in mechanical stimulation upon 3-day exposure to BPA 100 nM and 10 µM BPA, together with metabolical (increased lipid oxidation) and physiological (hyperplasia of epidermis, increased body wall thickness and ovarian atrophy) alterations [178]. On the other hand, also in *E. fetida*, BPS appeared to induce oxidative stress and the process of antioxidant defense in exposures up to 21 days at 1 and 10 mg/kg, altering the superoxide dismutase (SOD) and catalase (CAT) enzymatic activities, and also the expression profiles of the genes annetocin (ANN) and calreticulin (CRT) [170].

Cnidaria (*Hydra* sp.)

Hydra sp. are small freshwater dwelling organisms that belong to the phylum Cnidaria, usually inhabit slow running waters and ponds, attached to submerged subtracts (*e.g.* rocks, macrophytes), and are carnivorous, primarily feeding on small invertebrates. Though *Hydra* sp. occupy a low trophic level in the food web, acting as predator and prey, they play an important role in structuring the planktonic communities of ponds, being pointed out as a suitable bioindicator [179]. In fact, since the eighties, *Hydra sp.* has been used as model species to evaluate the hazards of environmental contaminants, being recognized as a relevant and sensitive model organism due to several advantageous characteristics [179–181]. Being a small organism, easy to culture in the laboratory, and with a fast reproduction and short generation time, it is appropriate for use in small-scale toxicity assays, allowing to easily test several chemicals and concentrations. *Hydra* sp. are diploblastic organisms (*i.e.* possess 2 cell layers: the outer ectoderm and the inner endoderm) facilitating the contact of all its cells with the contaminants present in the surrounding medium, contributing to the high sensitivity of these organisms to several types of chemicals. They have a high capacity to regenerate the whole body, which allows assessing teratogenic effects on stem cells. Adding to this endpoint, they can provide comprehensive toxicological analysis integrating several endpoints at individual level (e.g., morphology, feeding, budding, regeneration, reproduction), which constitute a gain when studying the hazards that bisphenols may pose to freshwater biota.

Only a few studies have addressed the ecotoxicity data of bisphenols in *Hydra* sp. species. To our knowledge, four studies have been published addressing the toxicity of BPA and BADGE to threespecies of Hydra: *H. vulgaris, H. magnipapillata*, and *H. oligactis*. Pascoe et al. (2002) [182] studied the effects of BPA

on *H. vulgaris*, recording that concentrations above 42 μg/L induced morphological changes in the hydras, and calculating a $LC_{50,96h}$ of 6.9 mg/L for this endpoint. Significant effects were also observed in the regeneration of hydra at 4.6 mg/L of BPA: sections of hydra could only regenerate tentacle buds. Later, Murgardas et al (2019) [183] reported that *H. magnipapillata* exposed to BPA for 96 h, also went through notorious morphological changes, calculating a slightly lower $LC_{50,96h}$ than the one reported by Pascoe et al. (2002) [182]: 5.137 mg/L. These authors also observed significant delays on head regeneration in hydras exposed to 15 μM (3.42 mg/L), hypothesizing that these effects were due to the cytotoxicity caused by BPA and the production of ROS at the amputated region which could promote DNA damage and cell death. Polyps exposed to this same BPA concentration also exhibited difficulties in capturing the prey, which could be related with failure in nematocysts discharge to paralyze the prey and on opening the mouth. The effect of BPA on the regeneration of gastric sections of this same species of hydra was studied by Park and Yeo (2012) [184]. Hydra sections exposed up to 5 ppm (5 mg/L) exhibited a regeneration process slower than those exposed in the control. Observations at the microscopic level revealed no injuries in the nematocysts, but cell apoptosis occurred in hydra exposed to BPA. After transferring the BPA-exposed hydras to the control medium, the authors observed that they recovered their regenerative capacity, and that the third generation showed a reproductive capacity similar to the ones from the control group. The effects of BPA, at similar concentrations as those tested by Park and Yeo (2012) [184] , on the reproduction of *H. oligactis,* was demonstrated by Fukuhori et al. (2005) [185]. Males and females of *H. oligactis* with sexual reproduction were exposed to concentrations of BPA ranging from 0.5 to 4 mg/L. In males, testis formation was suppressed at concentrations equal or above 1 mg/L, while tentacles and body length were reduced in organism exposed to 3 mg/L. As for females, the mean number of produced eggs was reduced at concentrations above 0.5 mg/L. Further 1 mg/L stimulated asexual reproduction, but budding production was suppressed at concentrations equal or above 2 mg/L. These authors also studied the accumulation of BPA in the body of hydras, having registered an influence of temperature. BPA concentration in the body of polyps was approximately 1.4 times higher at 10 °C than at 20 °C.

The toxicity of BADGE to *H. magnipapillata* was also reported by Park and Yeo (2012) [184]. Regarding the capacity for regeneration, BADGE induced similar effects as those observed for BPA and at the same concentrations. A reduction in the regeneration process of the hydra sections was observed, followed by a restoration of the regenerative capacity after the hydras being transferred to the control medium. Microscopic observations showed that exposure to BADGE caused apoptosis and cellular necrosis.

4.5.3. Aquatic vertebrates

Our investigation identified 17 BPA alternatives that were toxicologically evaluated on aquatic vertebrate models, specifically employing fish models, namely zebrafish (*Danio rerio*), Mozambique tilapia (*Oreochromis mossambicus*), African catfish (*Clarias gariepinus*), rainbow trout (*Oncorhynchus mykiss*), marine medaka (*Oryzias melastigma*), olive flounder (*Paralichthys olivaceus*), red common carp (*Cyprinus carpio*), rohu fish (*Labeo rohita*), European flounder (*Platichthys flesus*), goldfish (*Carassius auratus*), spotted snakehead (*Channa punctatus*), and brown trout (*Salmo trutta*) and amphibian models, namely Argentine toad (*Rhinella arenarum*), black-spotted pond frog (*Pelophylax nigromaculatus*; formerly *Rana nigromaculata*), Japanese wrinkled frog (*Rana rugosa*), African clawed frog (*Xenopus laevis)*, and western clawed frog (*Xenopus tropicalis*; formerly *Silurana tropicalis*); Supp. info. 6.

While many different vertebrate species were studied, zebrafish is by far the most comprehensively

studied aquatic vertebrate model organism in terms of the number of tested BPA alternatives as well as assessed endpoints and developmental stages (Supp. info. 6). Importantly, the collected studies show that BPA alternatives have not been systematically studied, *i.e.,* studies that evaluated specific endpoints (e.g., effects related to endocrine disruption) across the models (fish vs. amphibians), or multiple chemicals, are largely lacking. The different chemicals have also been unequally investigated so far, with nearly 70% of identified studies focusing on the top four BPA alternatives (BPS, BPF, BPAF, and TBBPA) (Fig.5). Among the chemicals that have been examined, BPS has undergone the most comprehensive research in terms of both the quantity of studies conducted and the range of endpoints explored.

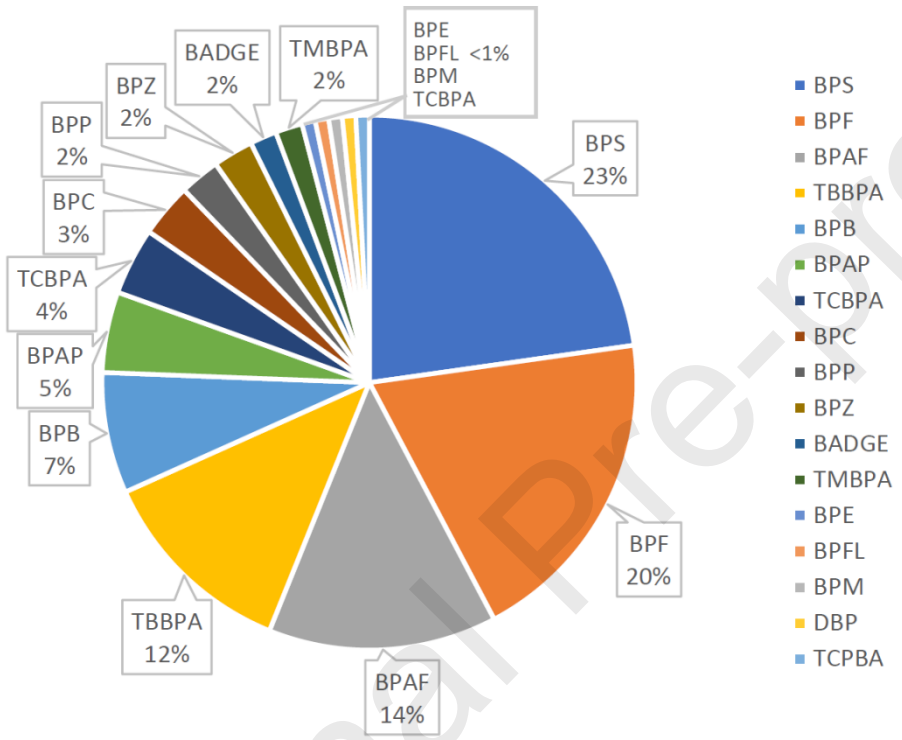

Fig. 5. Depiction of the proportions of the collected research studies utilizing aquatic vertebrate models (fish and amphibians) that examined various BPA alternatives. The percentage number also reflects the studies when the specific bisphenol was studied in mixtures. The details of the studies are provided in Supp. info 6.

Amphibian models

Compared to fishes, much less studies on BPA and its alternatives are available in amphibians (Supp. info 6). The summary of their effects is visualized in Supp. info. 7. They are restricted to the order *Anura*, whereas no experiments using other amphibian orders, *Urodeles* and *Caecilians*, have been reported. The available studies focused mainly on investigations of BPA effects on sexual differentiation and

development during early larval phases [186], demonstrating that already at 228 ng/L BPA can affect testicular development in *Xenopus laevis* tadpoles, by causing lacunae and testicular oocytes. In addition, BPA also affected growth and body weight; these were significantly increased in males exposed to concentrations of 2.28 µg/L BPA and above. This effect on growth could be explained by the induction of hepatic IGF-I (like growth factor 1) gene expression. Furthermore, a significant induction of VTG (vitellogenin) gene expression in the liver has been detected at 228 µg/L BPA.

Altogether, only five amphibian species, including *Xenopus laevis*, *Silurana tropicalis*, *Rhinella arenarum*, *Rana nigromaculatus*, and *Rana rugosa* have been used for investigating effects of BPA alternatives (BADGE, BPAF, BPB, BPF, BPS, DBD, TBBPA, TCBPA and TMBPA). The majority of these studies were using the established model organism *Xenopus laevis* to demonstrate endocrine disruptive effects. BADGE has been investigated only in *Rhinella arenarum* [187] at stages ranging from stage 3 to 25 exposed continuously at concentrations from 0.0001 to 25 mg/L revealing LC_{50} values for embryos and tadpoles with 0.04 and 2.2 mg/L, respectively. Pulsed exposures resulted in higher LC_{50} values from 0.58 to 14.9 mg/L, depending on the exposed developmental stage. In addition, sub-lethal effects were also observed in exposed organisms. Continuous exposure induced cell dissociation during embryo development at concentrations equal to or above 0.5 mg/L. In larvae exposed to a concentration of 10 mg/L or higher, neurological alterations were observed; those exposed to 15 g/L showed narcotic effects. Furthermore, after a 168 hour exposure to 5 mg/L, larvae exhibited signs of starvation, abnormal skin pigmentation, scare response to stimuli, and tail/axial flexures; while those exposed to 1 mg/L developed hydropsy and abnormal skin pigmentation. Regarding the observed sublethal effects after pulsed exposures, the main effects observed in embryos were microcephaly, hydropsy, axial flexures, and reduced body size, with a LOAEL of 1 mg/L.

In adult *R. nigromaculatus,* 14 d exposures to TCBPA and TBBPA ranging from 0.001 to 1 mg/L were performed and increased contents of alanine transaminase in the serum were observed in animals exposed from 0.001 to 0.1 mg/L of both compounds[188]. ROS contents in liver as well as expression of cytochrome C increased significantly after exposure to TCBPA and TBBPA, at all concentrations tested, demonstrating metabolic effects. *R. rugosa* tadpoles were exposed to TBBPA for 9 days at 544; 54.4, and 5.44 µg/L in the presence or absence of triiodothyronine (T3). T3-induced tail shrinking was inhibited by the lowest and highest tested concentrations [189], suggesting inhibitory or antagonistic modes of action concerning thyroid hormones. In addition, similar exposures with TBBPA, BPA, TCBPA, and TMBPA have been performed using *P. rugosa*, *P. tropicalis*, and X*. laevis* [190][191]. T3-induced tadpole tail shrinking in the wrinkled frog *R. rugosa* was suppressed by BPA, TBBPA, TCBPA, and TMBPA. BPA alternatives also inhibited spontaneous metamorphosis in the tropical clawed frog *S. tropicalis* controlled by endogenous circulating TH. These results indicate that BPA alternatives act as thyroid hormones (TH) antagonists. In *X. laevis* transgenic tadpoles carrying plasmid DNA containing TH response element (TRE) and 5'-upstream promoter region of the TH receptor (TR) βA1 gene linked to an enhanced green fluorescent protein (EGFP) gene, T3 induced a strong EGFP expression in the hind limbs. This induction was suppressed by BPA, TBBPA, TCBPA and TMBPA, suggesting BPA alternatives all act as antagonists to prevent the binding of T3 to TH receptor, resulting in inhibition of TR-mediated gene expression. In *X. laevis* tadpoles from stage NF 51, exposure for 21 d ranging from 2.5 to 500 µg/L TBBPA and short term exposure from 12 to 72 h with 0.1 and 1 µg/L T3 in the presence of 100, 250 and 500 µg/L TBBPA have been performed [192]. TBBPA inhibited development and reduced hind limb length at 500 µg/L after 21 d. Short term exposure to 100, 250 and 500 µg/L TBBPA inhibited T3-stimulated increase of thyroidal biomarkers TRß and b/ZIP gene expression, suggesting antagonistic modes of action on TH receptors as well.

Exposure to BPA and BPF ranging from 10^{-8} to 10^{-6} M in a *X. laevis* metamorphosis assay for 96 h [193]

revealed T3 induced brain morphological remodeling coupled with cell proliferation and neuronal differentiation, whereas both BPA and BPF in general antagonized T3 induced changes in a dose dependent manner with weak or no effects of bisphenols exposure alone. BPA and BPF interfered with TH signaling in *X. laevis* brains, especially in the presence of thyroid hormones (TH), and subsequently affected TH-dependent brain development. Further, similar experiments [194] investigating BPF with the T3 induced metamorphosis of *X. laevis* tadpoles exposed for 96 h at concentrations ranging from 10^{-8} to 10^{-5} M in the absence and presence of 10^{-9} M T3 revealed that higher concentrations of BPF (10^{-7} to 10^{-5} M) antagonized T3-induced TH response of gene transcription and morphological changes in a concentration-dependent manner whereas 10^{-9} M BPF exerted stimulatory effects on T-induced integral metamorphosis, displaying TH signaling disrupting effects with complicated concentration−response relationships. In the absence of T3, BPF inhibited development at metamorphic climax, but promoted pre- and pro-metamorphic development, displaying a developmental stage-dependent manner. Agonistic actions of BPF on Notch signaling in the intestine were observed, showing that BPF disrupts vertebrate development possibly via multiple pathways besides TH signaling.

X. laevis tadpoles at stages NF 45/46 (NF stages according to Zahn et al. (2022) [195] were exposed to BPB until stage NF 52 or until completion of metamorphosis from 10^{-8} to 10^{-6} M (to analyze whether potential effects of BPB on testis development are mediated by estrogen receptors). Therefore, tadpoles at stage NF 45/46 were exposed to 10^{-7} M BPB or the estrogen receptor antagonist ICI 182780, alone or in combination, until stage NF 52 [196]. Low concentrations of BPB disrupted testis differentiation partly via the estrogen receptor-mediated pathway and subsequently caused testicular dysgenesis after metamorphosis in *X. laevis*. Estrogenic chemicals resulted in inhibition of testis differentiation, which might later lead to testicular dysgenesis in frogs indicating adverse impacts of BPB on amphibian reproduction.

Early developmental effects of BPA and BPAF were investigated in *X. laevis* embryos at the two-cell stage 2 hours post-fertilization (hpf) (Arancio et al., 2019). BPA (10^{-6} to $5x10^{-5}$ M) and BPAF ($3x10^{-9}$ to $2.5x10^{-5}$ M) caused disrupted cleavage divisions, slowed cytokinesis, and cellular dissociation within 1–6 hours. Flexures of the spinal cord, shorter body axis/tail, craniofacial malformations, and significant mortality occurred with doses of BPAF (LC_{50} = $1.3x10^{-9}$ M). There were both shared and unique effects of all compounds, with BPAF having the greatest potency and toxicity (BPAF > BPA > estradiol). Further BPAF exposures ranging from 10^{-9} to 10^{-7} M have been performed using *X. laevis* tadpoles from stages NF 45/46 to 53 and 66 [197]. All concentrations of BPAF caused changes in testicular morphology at different developmental stages. Specifically, at stage NF 53, BPAF, like estradiol (E2), resulted in decreases in both, the size and the number of gonadal metameres (gonomeres), in testes, looking like ovaries. Some of the BPAF-treated testes remained segmented and even became discontinuous and fragmented at subsequent stages. Histological abnormalities were also observed in BPAF-treated testes, such as ovarian cavity at stages NF 53 and 66 and poorly developed seminiferous tubules 8 weeks post metamorphosis. At the molecular level, BPAF inhibited expression of genes highly expressed in testes of males at stage NF 53. Correspondingly, BPAF, like E2, inhibited cell proliferation in testes at stage NF 50. All results show that low concentrations of BPAF inhibited testicular differentiation and subsequent development in *X. laevis* by feminizing effects.

X. laevis tadpoles at stage NF 52 were exposed to BPA or BPF ranging from 10^{-8} to 10^{-6} M for 5 days [194]. BPA and BPF both significantly elevated Notch-related gene expression in a dose dependent manner. Cell proliferation and intestinal histological changes were observed. The study demonstrated that both, BPA and BPF, activate Notch signaling and subsequently disrupt intestinal development.

In summary, the rare studies dealing with BPA alternatives in amphibians suggest that BPA related compounds might similarly affect sexual differentiation by estrogenic or antiandrogenic modes of action. In addition, some compounds might also impact TH receptors in an antagonistic manner or even affect multiple yet unraveled metabolic pathways. Despite the fact that investigated BPA alternatives are often less effective than BPA or cause adverse effects, especially at very early developmental stages, only at relatively high concentrations, it has to be concluded that they are endocrine disruptive chemicals (EDCs) being effective in disrupting reproduction, development and metabolism.

Fish models

Upon reviewing the existing studies, we found that twelve different fish models were utilized to assess and characterize the bioactivity of BPA alternatives. In majority, the studies focused on examining the effects on early stages of zebrafish (Supp. info. 6). This preference for using zebrafish as a model aligns with other toxicological and pharmaceutical research, where the zebrafish model has a dominant presence due to its favorable experimental features as well as conservation of a significant number of morphological and physiological processes in vertebrate organogenesis [198]. With respect to BPA alternative research, the other fish species were typically employed to study only one or two chemicals. The studied BPA alternatives and specific endpoints evaluated in zebrafish are summarized in Supp. info 7.

Numerous studies have shown that BPA functions as an endocrine disruptor, capable of perturbing hormone levels and metabolism in vertebrates [4]. As a result, the primary emphasis of research on BPA alternatives has been on assessing their potential to interfere with the endocrine system. Many of the BPA alternatives demonstrate their influence on fish by affecting gene and hormone levels involved in reproduction, development, and growth. These studies reveal significant effects on gene expression associated with steroidogenesis and sex hormone production. Induction of vitellogenin, a precursor to egg yolk typically synthesized in females, was also observed in males, confirming the estrogenic effects of specific BPA alternatives (Fig.6). In addition to vitellogenin expression and production, in vitro and transgenic fish models have been also utilized to investigate the interaction with estrogen receptors and the modulation of estrogen-specific pathways. It has been shown that BPA and some of its substitutes (BPF, BPS, BPC, BPAF) have the capacity to interact with the different zebrafish ER subtypes in a selective manner. Hence in human or zebrafish cells expressing the zebrafish ER-subtypes alpha, beta1 or Beta 2, BPA and some bisphenol substitutes efficiently transactivate zebrafish estrogen subtypes, with a differential1 selectivity depending on the bisphenol tested and the zfER subtypes [199,200]. At the in vivo level the use of estrogeno-sensitive transgenic zebrafish embryo models such as tg(5xERE:GFP) and/or tg(cyp19a1b:GFP) allowed to demonstrate the capacity of BPA, BPS, BPF,BPC and BPAF to induce ER-signaling pathway in different tissue expressing ERs such as the heart, the liver and the brain [199–201]. The ability of bisphenols to induce brain aromatase in zebrafish seems a common feature as all the bisphenols tested up now are active in tg (cyp19a1b:GFP) (Christophe et al., under revision) [202]. Disruption of brain aromatase by bisphenols could play a role in their behavioral effect but a recent study showed that the behavioral changes (e.g. locomotor effect) measured in bisphenol-exposed zebrafish could not be linked to their potency to modulate brain aromatase expression [203]. These studies highlighted the relevance of combining cellular and whole-organism bioassays in zebrafish for the hazard assessment of bisphenols by providing relevant data on their mode of action on endocrine pathway and behavioral effect. From a regulatory point of view, the data obtained using these assays can not be used for risk assessment of these bisphenols. However, the reported mode of action and effect being similar to BPA they should be considered to accelerate the process of identifying these substitutes as endocrine

disruptors.

In some cases (*e.g.* BPS), we observed that there might be no need to evaluate its endocrine disruptive effects in any further detail, as the extensive evidence already available shows endocrine disruption potency on multiple levels. Thus, our findings highlight not only the importance of further toxicological research for less studied BPA alternatives but also demonstrate that some BPA alternatives have already been studied in sufficient detail to allow a reliable determination of their associated hazards, specifically connected with endocrine disruption.

BPA exposure has been shown to decrease the activity of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, which are responsible for neutralizing ROS (reactive oxygen species) and maintaining cellular redox balance [204]. As a consequence, the accumulation of ROS overwhelms the antioxidant defenses, resulting in oxidative stress and the potential disruption of various cellular processes in zebrafish. Oxidative stress appears to be a conserved effect observed in response not only to BPA but also to BPA alternatives. Similar to BPA, these alternatives can modulate levels of antioxidant enzymes and induce oxidative stress in various organisms, including zebrafish (Fig. 6.) and other investigated fish models (Supp. info 7) (e.g. African catfish, rainbow trout or marine medaka). Thus, oxidative stress appears to be a common pathway through which both BPA and its alternatives exert their toxic effects. This emphasizes the importance of considering oxidative stress as a critical aspect when evaluating the potential risks associated with these compounds.

With respect to BPA alternatives, the various adverse outcomes have been studied in a rather non-systematic way. For many of the chemicals, and in contrast to endocrine disruptive potency, many of the endpoints are studied less frequently, for example, the immunity and microbiome. The microbiome and immune system, although crucial aspects of an organism's health, have been overlooked as endpoints in the study of BPA alternatives. While the effects of BPA on the microbiome and immunity have been investigated [205,206], only one study examined the impact of BPA alternatives on these endpoints [207]. The microbiome, consisting of the diverse community of microorganisms residing in the body, plays a vital role in various physiological processes, including immune function [208]. Disruptions to the microbiome can have far-reaching consequences for overall health and immune system regulation [209]. Thus, by neglecting to assess the effects of BPA alternatives on the microbiome and immune system, our understanding of their potential health risks on aquatic vertebrates remains incomplete. It is essential to broaden the scope of research to include these critical endpoints to comprehensively evaluate the environmental safety and biological impacts of BPA alternatives.

In conclusion, the summary of the effects of BPA alternatives on aquatic vertebrates indicates that some of the chemicals can be hazardous, sharing the mode of action with BPA (e.g. deregulation of endocrine system and induction of oxidative stress). Future research should focus on exploring overlooked endpoints, as well as investigating the long-term effects and potential ecological implications of less studied BPA alternatives on fish populations and aquatic ecosystems. Further, for certain BPA alternatives (*e.g.* BPS), the existing scientific studies have already provided extensive evidence regarding their potential dangers to the environment. Therefore, there is a strong rationale to assert that conducting additional toxicological studies on these alternatives would be redundant and unnecessary. Decision makers are now faced with a responsibility to take swift and decisive action based on the available research data. It is imperative that they prioritize the protection of the environment and public health by implementing appropriate measures to regulate or restrict the use of identified hazardous BPA alternatives. By taking proactive steps based on the existing knowledge, decision makers can effectively mitigate the potential risks associated with these substances and safeguard the well-being of ecosystems and living organisms.

Model organism	Impact on	Impacted endpoint	BPS	BPF	BPAF	BPB	BPAP	BPZ	BPC	DBP	BPE	BPM	BPP	BPFL	BADGE	TBBPA	TCBPA	TMBPA
Zebrafish (*Danio rerio*)																		
Early life stages	Development	Hatch rate	Y/N	Y/N	Y/N	Y/N	Y/N		Y		Y							
		Survival rate / mortality	Y/N	Y/N	Y/N	Y	Y	Y	Y		Y	Y		Y				
		Growth (length)	Y/N	Y/N	Y/N	Y												
		Yolk consumption	Y															
		Lipid composition	Y															
		Endoplasmatic reticulum	Y															
		Body weight														Y	Y	
		Deformities / Morphology		Y	Y/N	Y/N	N		Y		Y							
		Loss of pigmentation		Y														
	Oxidative stress	ROS	Y	Y	Y/N	Y	N				Y							
		Enzymes activity	Y	Y	Y	Y	Y	Y	Y		Y							
	Immunity	Immunity markers	Y	Y	Y													
	Neurotoxicity	Locomotion activity	Y	Y	Y	Y/N	Y		Y		Y					Y/N	Y	
		Neurogenesis/Neurodevelopment	Y/N	Y/N	Y	Y	Y									Y	Y	
		Neurotransmitters	Y	Y	Y													
		Brain structure		Y		Y												
		Behavior			Y		N									Y/N		
		Ache activity			Y												Y	
		Microglia, Astrocytes		Y														
		Neuroinflammatory response		Y														
	Endocrine disruption	Thyroid hormones				Y	Y											
		Estrogen Receptor	Y	Y				Y	Y									
		Insulin signaling		Y	Y													
		Testosterone				Y												
		Estrogen (ER alfa)	N	Y	Y	Y												
		Vitellogenin (Vtg)		Y	Y	Y	N		N		Y	N	Y	Y				
		Antiestrogen effect										Y	Y	Y				
	Sensory endpoints	Retinal structure	Y															
	GI tract	Intestinal Integrity		Y														
	Apoptosis			Y	Y	Y	Y		Y		Y							
	Cardiac endpoints	Heart rate, Heartbeats	Y	Y	Y/N	Y	N	Y	Y		Y					Y	Y	
		Loss of blood vessels															Y	
	Metabolic	Glucometabolic		Y	Y													
Zebrafish (*Danio rerio*)																		
Adult stages	Microbiome	Community structure	Y	Y	N	N												
		Diversity indexes	Y	Y/N	N	N												
		Microbiome function	Y	Y	N	N												
	Development	Growth (length)	Y	Y														
		Body weight	Y	Y														
	Neurotoxicity	Locomotion activity		Y														
		Social behavior	Y															
		Anxiety response	Y															
	Endocrine disruption	Estrogen receptor levels	Y	Y				Y	Y									
		Steroidogenic enzymes	Y															
		Vitellogenin (vtg)	Y	Y														
		17b estradiol (E2)	Y	Y														
		Thyroxine	Y															
		Triiodothyronine	Y															
		Testosterone	Y	Y														
	Oxidative stress	ROS / oxidative stress	Y	Y				Y	Y									
		Enzymes activity	Y	Y				Y	Y									
	DNA damage	DNA methylation	Y															
	Reproduction	Reproductive capacity (males)	Y															
		Gonadosomatic index	Y															
		Sperm count	Y															
		Egg production	Y			Y												
		Ovarian and testicular histology		Y														
	Transgenerational effect	DNA methylation in offsprings	Y															
		Alter social behavior	Y															
	Hepatotoxicity	Hepatosomatic index	Y															
		Fatty acid synthesis/degradation	Y															
		Fat / TAG accumulation	Y															

Fig.6. The overview of compiled research utilizing zebrafish as a model organism. The table shows what BPA alternatives are active with respect to the examined endpoints (Y- studied endpoint interacts with chemical, N- studied endpoint does NOT interact with chemical, Y/N- the studies show both trends). The studies used for this figure are described in detail in Supp. info. 6. The overview of compiled research for other studied fish models is presented in Supp. info. 7.

5. Conclusion and future perspectives

BPA and BPA alternatives are chemicals commonly found in all environmental compartments. Although BPA is currently regulated and its use is restricted in many products, BPA can still be found in the environment at a relatively high level in comparison with its alternatives. However, it can be expected that levels of BPA alternatives will dominate in future. Therefore, the monitoring and surveillance of water and sediment quality should be enhanced to include measurements of BPA alternatives to monitor their levels and ensure development of timely mitigation strategies.

The review on QSAR brings attention to two primary concerns. Firstly, there is a notable inadequacy in the representation of bisphenols within the training datasets, coupled with a scarcity of experimental data available for compounds related to bisphenols. Secondly, there exists a considerable demand for comprehensive *in silico* methods that encompass the entire spectrum of bisphenols, including their derivatives and alternative compounds. This demand arises from the significance of certain endpoints, which also carry regulatory importance. Within the toxicokinetic aspect of this review, the dynamic models analyzed suggest potential variations in ADME (absorption, distribution, metabolism, and excretion) processes among bisphenol analogues. This calls for further research to validate these findings. On the toxicodynamic aspect, this review underlines the necessity for predictive models capable of assessing the effects of bisphenols across various levels of biological organization, ranging from individual organisms to entire ecosystems.

When comparing BPA to its alternatives, it becomes evident that there has been less extensive research into the assessment of the biological activities of BPA alternatives. However, these BPA alternatives are indeed present in the environment, their effects have not been adequately examined through controlled experiments that prioritize specific bisphenols, such as PBS. The available toxicological studies nevertheless cover experimental models from many parts of the phylogenetic tree, starting with microorganisms to vertebrates. In terms of the impact on microbiome, the studies emphasize the substantial impact that both BPA and its alternatives can exert on microbial organisms and communities, disrupting their physiology, operations, and composition. This could potentially have an impact for hosts and the functions of ecosystems and the services they provide. The chapters promote the idea of including microbes and their communities as integral components of conventional hazard assessment processes, specifically for BPA alternatives. Our investigation also delved into the significance of exploring the endocrine-disrupting effects of both BPA and its alternatives on invertebrates, with a particular focus on arthropods and insects. We stressed the importance of conducting more comprehensive assessments that go beyond the traditional reproductive studies. These investigations reveal that BPA and its alternatives can indeed interfere with the endocrine systems of insects, particularly by disrupting the ecdysis process and potentially causing genotoxic effects. This underscores the necessity for utilizing advanced molecular, analytical, and omics techniques to pinpoint molecular markers that can be used to evaluate potential adverse impacts on the insect endocrine systems. Furthermore, it highlights the need for developing new AOPs that are specifically related to the ecdysis processes in invertebrates.

The presence of BPA and its alternatives in mollusks found in the environment stresses the need to incorporate mollusks into future research efforts. Although the effects of BPA on mollusks have been thoroughly examined, there is a shortage of research regarding the impact of BPA alternatives on these organisms. Consequently, it is vital to conduct additional investigations in this domain, particularly focusing on the early life stages of mollusks. Ascidians were another class of organisms used to study the endocrine effect of BPA. However, none BPA alternatives were studied (only BPA), using this model, the studies show ascidians as valuable model organisms for studying endocrine-disrupting chemicals due to their close evolutionary proximity to vertebrates and various advantageous characteristics for laboratory studies. Similarly, crustaceans (*e.g.*, Daphnia spp., Artemia spp.) are a valuable model organism for evaluating the impacts of environmental pollutants, including endocrine-disrupting chemicals. It might be concluded that most of the available data refers to BPA and the information about the toxicity of BPA alternatives is very inconsistent and limited to specific endpoints including neuro-endocrine axis. Earthworms (phylum Nematoda) provide valuable insights into the toxic effects of BPA and its alternatives. BPA alternatives impact a broad spectrum of biological systems in earthworms exhibiting effects in growth, reproduction, behavior, and metabolism. Interestingly, *C. elegans* were successfully used for investigation of obesogenic potency of BPA alternatives such as BPS. Cnidaria (Hydra spp.) display varying degrees of sensitivity to bisphenols like BPA and BADGE which shows that BPA alternatives can be sensed by this family of organisms. This underscores the need for further research on the ecotoxicity of bisphenols in these organisms, considering their ecological significance in freshwater ecosystems.

The impact of BPA alternatives on higher organisms were also studied using vertebrate models. For studies employing vertebrate models, we found that current studies cover only selected BPA alternatives and thus have not been systematically studied (nearly 70% of identified studies focused on the top four BPA alternatives (BPS, BPF, BPAF, and TBBPA)). Specifically, the existing studies on amphibians, particularly in the order Anura, demonstrate that bisphenols, including BPA and its alternatives, can disrupt sexual differentiation, development, metabolism and endocrine systems in various amphibian species. Similarly for fish studies, research primarily focused on impact on endocrine systems. In fish, BPA alternatives can disrupt gene and hormone levels associated with reproduction, development, and growth, and induce oxidative stress.

In summary, to ensure timely mitigation strategies, future monitoring and surveillance of the quality of the environment should be enhanced to include measurements of BPA alternatives and monitor their levels closely. The biological activity has been studied for BPA alternatives, but in a non-systematic way and prioritizing only a limited number of chemicals. Nonetheless, for several common BPA alternatives, extensive scientific research has already provided substantial evidence regarding their potential harm to the environment. The collective assessment of BPA alternatives suggests that certain chemicals may pose hazards, mirroring the mode of action seen with BPA, such as endocrine system disruption and oxidative stress induction. Therefore, it is reasonable to argue that carrying out further toxicological investigations on specific well-studied BPA alternatives would be superfluous and unwarranted. In terms of studied endpoints, several key biological systems are overlooked including the impact on immunity. Future research should emphasize unexplored aspects, the possible adverse effects caused by the exposure to mixtures of bisphenols at realistic environmental concentrations and the long-term consequences on ecosystem.

In the frame of project PARC (work package 5 (WP5)) and more specifically in the frame of internal project “BPA alternatives and associated mixtures (Data gaps and NAM development)” we will focus on the investigation of potential adverse effects of certain individual BPA alternatives and “real-life” mixtures on different species to fill the identified gaps regarding the potential harmful effects of bisphenols in the

environment (activity of sub-task 5.1.2.). In addition, within this project, new technologies will be developed, such as *in silico* and modeling tools, read-across methodologies, and other advanced new approach methodologies (NAMs) covering both invertebrate and vertebrate organisms and several endpoints such as endocrine disruption (including metabolic disruption), non-genotoxic carcinogenicity, (developmental) neurotoxicity, immunotoxicity (activity of sub-task 5.2.2.).

In PARC, complementary to environmental related studies, BPA alternatives are also investigated in more human-oriented *in vitro* studies. The goal is to assess the hazards associated with these alternatives, contributing to their regulation as part of sub-task 5.1.1. This project aims to produce a complete dataset on the immunotoxicity, endocrine disruption activity, neurodevelopmental toxicity, genotoxicity, and non-genotoxic carcinogenicity of BPA alternatives and to explore mechanisms underlying the effects observed that would feed into adverse outcome pathways. The results of these projects, as well as the results of other projects within PARC including monitoring activities of BPA alternatives, can be exploited by decision makers and risk managers at European level. Decision makers bear the responsibility of action based on existing research data. Their key responsibility is to prioritize environmental and public health protection by implementing appropriate measures to regulate or restrict the use of identified hazardous BPA alternatives. Proactive measures grounded in current knowledge can effectively mitigate potential risks, ensuring the well-being of ecosystems and living organisms.

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Declaration of interests

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: