Partnership for the Assessment of Risks from Chemicals

Deliverable D5.4 1st closing data gaps report

WP5 – Task 5.1

PARC Partnership for the Assessment of Risks from Chemicals

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Abstract

This deliverable summarizes the preliminary results of the PARC WP5 Task 5.1, aiming to close key data gaps for substances of emerging concern. Focus is placed on two major substance groups: natural toxins and bisphenol A (BPA) alternatives, addressing both human and environmental health. For human health, prioritized mycotoxins (enniatins including beauvericin and Alternaria toxins) were tested across genotoxicity, endocrine disruption, developmental neurotoxicity, and immunotoxicity endpoints, employing both OECD test guidelines and new approach methodologies (NAMs). In a parallel project, hazard testing of BPA alternatives examined metabolism, bioactivation, and toxicological profiles using as well OECD test guidelines and in vitro systems aligned with regulatory relevance.

On the environmental side, aquatic organisms such as Daphnia magna, Lymnaea stagnalis, and Chlorella vulgaris were used to assess the ecotoxicity of selected natural toxins and BPA alternatives under standard OECD and ISO guidelines. Both single and mixture exposures were tested, revealing species- and compound-specific effects and emphasizing the relevance of temperature and combined stressors in ecological risk assessment.

Altogether, this first data gap report contributes to strengthening risk assessment capabilities across the EU by supporting better prioritization and regulation of under-studied substances. The data are being shared and discussed with regulatory agencies (EFSA, ECHA, EEA), and further testing is ongoing to complete hazard characterizations.

Key Words

Natural toxins, BPA alternatives, genotoxicity, endocrine disruptive effects, immunotoxicity, developmental neurotoxicity (DNT), ecotoxicology, *in vitro*, risk assessment, new approach methodologies (NAMs)

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Authors and Acknowledgements

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Acronyms

ALT – Altenuene

AME – Alternariol Monomethyl Ether

AOH – Alternariol

AOP – Adverse Outcome Pathway

AR SSTA – Androgen Receptor Steroidogenesis and Transcriptional Activation Assay

ATX-I – Altertoxin I

BEA – Beauvericin

BMC30 – Benchmark Concentration for 30% response

BPA – Bisphenol A

BPAF – Bisphenol AF

BPAP – Bisphenol AP

BPB – Bisphenol B

BPE – Bisphenol E

BPF – Bisphenol F

BPP – Bisphenol P

BPPH – Bisphenol P Hydroquinone

BPZ – Bisphenol Z

BPS – Bisphenol S

BPS-MAE – Bisphenol S-MAE

BPS-MPE – Bisphenol S-MPECaco-2 – Human Colorectal Adenocarcinoma Cells

CYN – Cylindrospermopsin

DA – Domoic Acid

DHT – Dihydrotestosterone

DMSO – Dimethyl Sulfoxide

DNT – Developmental Neurotoxicity

DON – Deoxynivalenol

E-Screen Assay – Estrogenic Activity Assay in MCF-7 Cells

EC50 – Effective Concentration for 50% effect

ENNs – Enniatins

ER SSTA – Estrogen Receptor Steroidogenesis and Transcriptional Activation Assay

ETS – Electron Transport System

γH2Ax Assay – Phosphorylation Assay for DNA Damage Response

GR – Glucocorticoid Receptor

HABs – Harmful Algal Blooms

HCEC-1CT – Human Colonic Epithelial Cells (non-tumorigenic)

HepG2 – Human Liver Cancer Cell Line

hiPSC(s) – Human Induced Pluripotent Stem Cell(s)

H-NMR – Proton Nuclear Magnetic Resonance

HPLC-UV – High-Performance Liquid Chromatography with Ultraviolet Detection

HPRT Assay – Hypoxanthine-Guanine Phosphoribosyltransferase Assay

IC50 – Half-Maximal Inhibitory Concentration (a measure of a substance's potency)

IL-1β, IL-2, IL-6, IL-8, IL-10 – Interleukins (cytokines)

JHASA – Juvenile Hormone Activity Screening Assay

LC50 – Lethal Concentration for 50% effect

LDH – Lactate Dehydrogenase (used in cytotoxicity assays)

LOAEL – Lowest Observed Adverse Effect Level

LPS – Lipopolysaccharide

MAP2, ELAVL2, GRIK4 – Neuronal markers

MC-LR – Microcystin-LR

MCT8 – Monocarboxylate Transporter 8

MCT8 transporter – Monocarboxylate transporter 8

NAMs – New Approach Methodologies

NF-κB – Nuclear Factor Kappa B

NIS – Sodium/Iodide Symporter

NIS transporter – Sodium iodide symporter

NPC(s) – Neural Progenitor Cell(s)

PBPK – Physiologically Based Pharmacokinetic

PSD95, gephyrin – Synaptic plasticity markers

QSAR – Quantitative Structure-Activity Relationship

RT-qPCR / qRT-PCR – Quantitative Reverse Transcription Polymerase Chain Reaction

RTgill-W1 – Fish Cell Line Acute Toxicity using the RTgill-W1 cell line

SAR Analysis – Structure-Activity Relationship Analysis

SoM Scoring – Site of Metabolism Scoring

SOP – Standard Operating Procedure

STX – Saxitoxin

STTX: Stemphyltoxin III

T – Testosterone

T3 – Triiodothyronine (thyroid hormone)

TBA – Terbuthylazine

TCR – T-cell Receptor

TDAR – T Cell–Dependent Antibody Response

TeA – Tenuazonic Acid

TEN – Tentoxin

TDI – Tolerable Daily Intake

TNF-α – Tumor Necrosis Factor Alpha

TH – Thyroid Hormone

YTX – Yessotoxin

ZEN – Zearalenone

Introduction

PARC task 5.1 Closing data gaps of concern aims to investigate and close existing data gaps through toxicity testing. Following a prioritization exercise and further consultation with partners involved in hazard and risk assessment in national and European agencies, the following groups of substances have been selected for the first round of testing: natural toxins as well as alternatives to bisphenol A (BPA alternatives) [Marx-Stoelting et al., 2023].

Under T5.1, several projects have been initiated to fill regulatory gaps in hazard assessment, following consultations with EU agencies (Table 1).

Table 1: Outline of the projects approved under PARC WP5- T5.1 Data Gaps

Project		Status
P51.1.a Toxins	Hazard identification and hazard characterisation of the mycotoxins enniatins including beauvericin and *Alternaria* toxins in order to close data gaps and improve risk assessment for human health	05/2022 – 04/2026 Preliminary data was obtained and further studies are ongoing
P5.1.1.b BPA HumTox	Hazard assessment of bisphenol A alternatives to close data gaps of concern for human health and improve their risk assessment	05/ 2022 – 10/2026 Preliminary data was obtained and further studies are ongoing
P5.1.1.c TG+ EnnB1	OECD test guideline-conform animal study with additional endpoint omics-enhanced in vivo study on Enniatin B1	05/2024 – 04/2027 Studies will be initiated by CRO in May 2025 Additional analysis will be performed by selected PARC partners
P5.1.1.d PlasticLeach	Hazard characterization of leachable chemicals present in plastics	05/2025 – 01/2029
P5.1.2.a Natural toxins aqua	Toxicity assessment of naturally occurring toxins on aquatic organisms	05/2022 - 04/2025 First data gaps are filled and project will be finished
P5.1.2.b BPA alternatives	Adverse effects of individual compounds and mixtures of BPA alternatives on organisms from different taxa	05/2022 – 04/2026 Preliminary data was obtained and further studies are ongoing

This report aims to provide an overview of the preliminary results from PARC WP5 Task 5.1 on data gaps proving information on the first results obtained to close the prioritized data gaps (human health and environment health), the scientific achievements (publications, oral presentations etc.) and the interactions developed with appropriate regulators (e.g. EU Agencies).

Part I of this deliverable focuses on natural toxins. We will present data from project P5.1.1.a, which examines human toxicity, and project P5.1.2.a, which investigates environmental effects.

Part II of the deliverable follows a similar structure, presenting data on BPA alternatives. Specifically, data from project P5.1.1.b, focusing on human health, and project P5.1.2.b, which focuses at environmental toxicity, will be shared.

Part I: Natural toxins: Human Health & Environment

1. Introduction

Natural toxins have no manufacturer or supplier that is responsible for providing hazard data. Due to climate changes, the human exposure to natural toxins will likely increase (Gobler, 2020) and regulatory agencies have asked for hazard data to improve the risk assessment. This will ultimately result in recommendations on which

natural toxins should be closely monitored in order to prevent adverse human, animal and environmental health effects. As such, different classes of natural toxins are in the scope for filling the gaps on hazard assessment, including phycotoxins, phytotoxins and mycotoxins.

In terms of human health, two classes of emerging mycotoxins, enniatins including beauvericin and *Alternaria* toxins were pointed to be of regulatory need for more specific hazard data, requiring data performed according to OECD test guidelines, when possible, or for specific endpoints using new approach methodologies (NAMs).

For environmental health, the major classes of toxins are those produced by harmful algal blooms (HAB)s (phycotoxins). Hazard data for these toxins and in particular for aquatic invertebrates and microalgae are lacking. These data are needed to support EU guidelines to achieve good environmental status for marine and freshwater environments (marine framework directive and water framework directive.

A paper on hazard identification of enniatins and beauvericin to identify data gaps and improve risk assessment for human health was published in Archives of Toxicology. https://doi.org/10.1007/s00204-025-03988-3

A paper on hazard characterisation of *Alternaria* toxins to identify data gaps and improve risk assessment for human health was published in Archives of Toxicology. https://doi.org/10.1007/s00204-023-03636-8

Regular meetings with EFSA were held to present plans and preliminary results for the human health part.

In the upcoming months, these preliminary results are expected to be consolidated, and the full sets of data will be available to answer regulatory challenges.

2. Human Health effects of Natural toxins

Within this project, 17 PARC partners contribute to the efforts to close crucial data gaps for ENNs, BEA and *Alternaria* toxins for human health. Respective data gaps have been identified (Figure 1) and summarized in two reviews, one focused on the hazard characterization of *Alternaria* toxins [Louro et al., 2024] and one review on the data gaps for ENNs and BEA [Behr et al., 2025]. The focus of the natural toxins project is on closing data gaps on genotoxicity, immunotoxicity and endocrine disruption, for hazard characterization of two important classes of emerging mycotoxins: enniatins including beauvericin and *Alternaria* toxins to improve the risk assessment. Enniatins (ENNs) are mycotoxins produced by various *Fusarium* species. From the more than 30 known enniatin analogues, ENN A, A1, B, B1 and beauvericin (BEA) are the most abundant in grain and grain-based products. *Alternaria* toxins, formed by *Alternaria spp*, especially *Alternaria alternata*, comprise a broad spectrum of more than 70 identified secondary metabolites. *Alternaria* spp. are able to infect a spectrum of substrates including tomatoes, apples, berries or grains and grow under a wide range of climatic conditions (Schamey et al., 2024; Pavicich et al., 2022). Tenuazonic acid (TeA), alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT) and tentoxin (TEN) appear to be the most abundant *Alternaria* toxins in food and feed, but occurrence data are still limited. Little is known on the occurrence of *Alternaria* toxins out of the class of perylene quinones. Altertoxin I (ATX-I) is the only perylene quinone formed by *Alternaria alternata* which is commercially available in sufficient amounts and purity to allow hazard characterization.

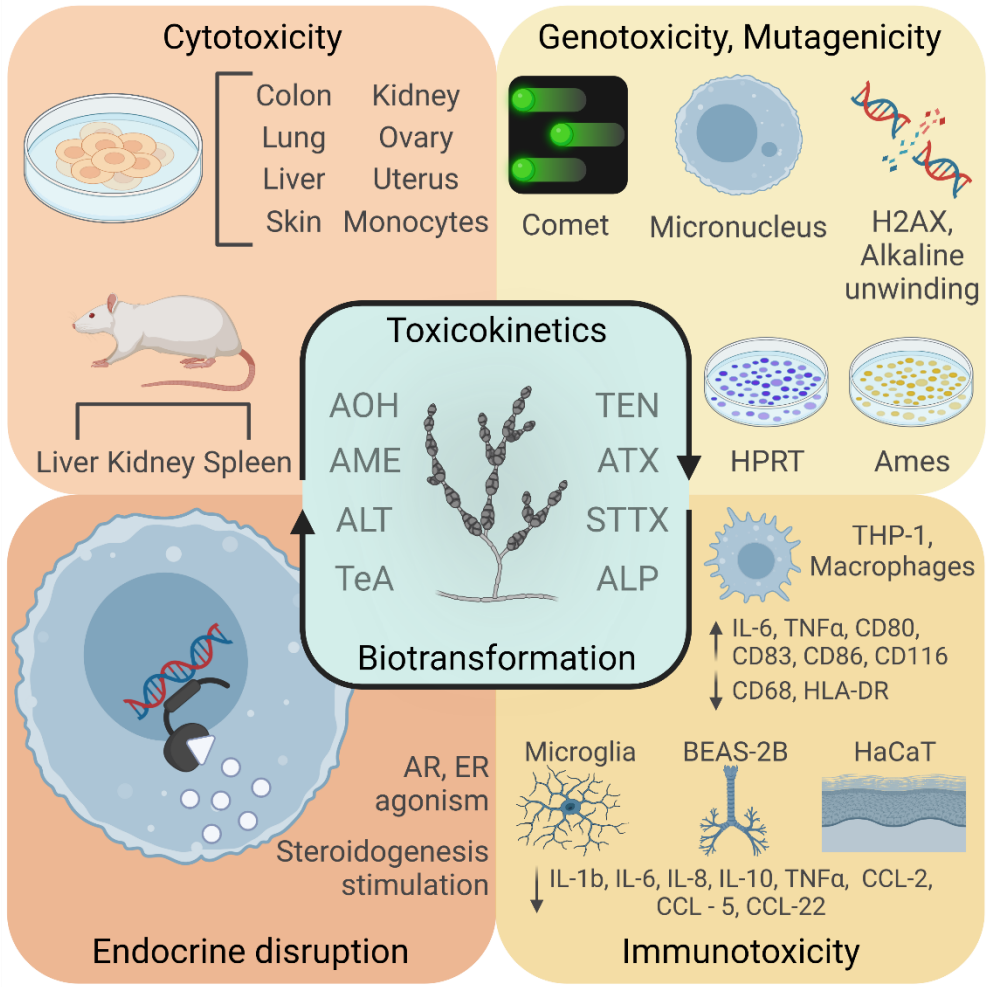


Figure 1: Overview of endpoints reviewed and data gaps identified for *Alternaria* toxins. Data gaps are being addressed under WP5.1.1.a (Credits of image to Francesco Crudo, UNIVIE, STTX: Stemphyltoxin III)

2.1. Substances

ENNs and AME were chemically synthesized by Technical University of Berlin [TUB (DE)]; the synthesis of AOH is still ongoing. The other test substances were commercially acquired. To avoid batch-to-batch variations, a centralized order was arranged by BfR (DE). The purity of the ENNs was confirmed by NVI (NO) and the purity of AME by UNIVIE (AT). Solubility studies were performed for the enniatins that were selected for further study (ENN A, A1, B and B1) by UNAV (ES). A Standard Operating Procedure (SOP) for the handling, storage and stability was prepared for both ENNs including BEA and *Alternaria* toxins and are constantly updated by the partners.

A list of the substances studied in this project is presented in Table 2 and the respective purity and solubility information in Table 3.

Table 2: List of mycotoxins investigated in P5.1.1.a

Substance		Abbreviation	CAS no.	Molecular weight (g/mol)	Structure
Enniatins	**Enniatin A**	ENN A	2503-13-1	681,9	$C_{36}H_{63}N_3O_9$
	Enniatin A1	ENN A1	4530-21-6	667,9	$C_{35}H_{61}N_3O_9$
	Enniatin B	ENN B	917-13-5	639,8	$C_{33}H_{57}N_3O_9$
	Enniatin B1	ENN B1	19914-20-6	653,8	$C_{34}H_{59}N_3O_9$

	Beauvericin	BEA	26048-05-5	784,0	$C_{45}H_{57}N_3O_9$
***Alternaria* toxins**	**Alternariol**	AOH	641-38-3	258,2	$C_{14}H_{10}O_5$
	Alternariol monomethyl ether	AME	23452-05-3	272,3	$C_{15}H_{12}O_5$
	Altertoxin I	ATX-I	56258-32-3	352,3	$C_{20}H_{16}O_6$
	(-)-Altenuene	ALT	889101-41-1	292,3	$C_{15}H_{16}O_6$
	Tenuazonic acid	TeA	610-88-8	197,2	$C_{10}H_{15}NO_3$
	Tentoxin	TEN	28540-82-1	414,5	$C_{22}H_{30}N_4O_4$

Table 3 gives details of the supplier, purity and solubility properties measured in the different substances.

Table 3: Purity and solubility information for ENNs and *Alternaria* toxins

	Substance	Supplier	Purity	Solubility
Enniatins	**ENN A**	TU Berlin	100%	Max. solubility (1): 33,6 mg/mL
	ENN A1	TU Berlin	100%	Max. solubility (1): 31,3 mg/mL
	ENN B	TU Berlin	100%	Max. solubility (1): 60,6 mg/mL
	ENN B1	TU Berlin	100%	Max. solubility (1): 100,0 mg/mL
	BEA	Cayman chemicals	100%	pending
***Alternaria* toxins**	**AOH**	TU Berlin	pending	20 mg/mL and 30 mg/mL (2)
	AME	TU Berlin	100%	10 mg/mL (2)
	ATX-I	Cayman chemicals	95,9%	10 mg/mL (2)
	ALT	Cayman chemicals	99%	10 mg/mL (2)
	TeA	SantaCruz	95%	20 mg/mL and 50 mg/mL (2)
	TEN	Cayman chemicals	98,9%	10 mg/mL (2)

(1) Tested in a solubility test by UNAV (ES)
(2) Stated solubility acc. Supplier, not the maximum solubility

2.2. Endpoints and performed and planned studies

In this section a summary of the different endpoints and performed and planned studies is presented. More detailed description of the methods can be found in the Annex of this deliverable.

Genotoxicity and mutagenicity

The assessment of genotoxic effects is mandatory in the risk assessment of chemicals in several sectors as genotoxicity may lead to adverse effects including cancer, aging, and genetic diseases. According to previously identified data gaps, the selected toxins were tested for genotoxicity and mutagenicity with special emphasis on OECD-conform methodology.

The **Ames assay** (according OECD TG 471) was conducted to assess the mutagenic potential of the ENN analogues A, A1, B and B1 [BfR (DE)] and *Alternaria* toxins [NIB (SI)] by measuring their ability to induce frameshift and/or point mutations in specific strains of bacteria. Due to a recently published opinion by EFSA classifying BEA as non-genotoxic, BEA was not included in the Ames assay (EFSA 2024). An *in vitro* mammalian cell **micronucleus Assay** was performed for ENNs, BEA and *Alternaria* toxins according to the OECD test guideline 487 in TK6 cells [Sciensano (BE)] as well as in HepG2 cells on *Alternaria* toxins [INSA (PT)]. In addition, two indicator tests were performed to assess the genotoxic potential of ENNs, BEA and *Alternaria* toxins: A **SOS/umu test** was used to detect the induction of the SOS response in bacteria, which occurs when DNA damage is present [UNAV (ES)]. The **γH2Ax Assay** was conducted to assess the phosphorylation of γH2Ax which is an early event in response to double strand breaks [BfR (DE)]. Depending on the outcome of the beforementioned assays, a HPRT assay with *Alternaria* toxins and a Comet assay with ENNs may be planned.

Endocrine effects

The endocrine system plays a crucial role in regulating various physiological processes. Because it relies on precise communication between organs, any disruption can have widespread effects on the entire organism. Several *Alternaria* toxins including alternariol and its monomethyl ether have already been reported to act as ligands of human estrogen receptors (DellafioraDellafiora et al., 2018). However, so far data on endocrine disruptive activity using OECD-conform test methodology are still missing. In this context, we will perform *in vitro* studies examining the effects of ENNs including BEA and *Alternaria* toxins on sex hormone receptors and steroidogenesis.

The **ER SSTA** (Estrogen Receptor Steroidogenesis and Transcriptional Activation Assay) [INRS (FR)] and the **AR SSTA** (Androgen Receptor Steroidogenesis and Transcriptional Activation Assay) [BPI (GR)] are *in vitro* tests used to assess the interaction with the estrogen receptor (ER) and androgen receptor (AR). These assays measure the ability of a substance to bind to these hormone receptors and activate gene expression, providing insights into potential hormonal disruptions. The tests were performed according to the OECD TG 455 and TG 458, respectively. The *in vitro* Human **H295R Steroidogenesis Assay** (OECD TG 456) was performed to assess the endocrine potential of ENNs and BEA [ISS (IT)] and *Alternaria* toxins [BfR (DE)] to alter the synthesis of the sex steroid hormones 17β-estradiol (E2) and testosterone. In addition, several assays investigating the impact of ENNs and *Alternaria* toxins on the molecular initiating events in the **thyroid metabolism** are performed [BfR (DE)]. Therefore, functional assays for the activation of sodium-iodide-symporter (NIS), thyreoperoxidase (TPO), deiodinase 1 (DIO1) and iodothyrosine deiodinase 1 (DEHAL1) are used. To measure the binding properties on thyroid receptor α, a dual-luciferase reporter gene assay will be established. An **E-Screen assay** in MCF-7 cells investigating the estrogenic potential is also planned for *Alternaria* toxins [BPI (GR)]. Depending on the outcome of the previous studies, the E-Screen assay will also be performed on ENNs and BEA [BfR (DE)].

Immunotoxic effects

Mycotoxins can disrupt the immune function, leading to immunosuppression, inflammation, or affect mechanisms in the development of allergic response or autoimmune disease. These effects increase susceptibility to infections and can result in other health risks. Understanding mycotoxin-induced immunotoxicity is essential for assessing their full impact and regulatory significance. Therefore, several *in vitro* studies investigating the potential of ENNs, BEA and *Alternaria* toxins on immune system function will be performed.

The **NF-κB reporter gene assay** was used to investigate how ENNs, BEA and *Alternaria* toxins influence the NF-κB signaling pathway in THP-1 Lucia™ cells [UNIVIE (AT)]. NF-κB is a critical transcription factor that controls a wide range of processes, including immune responses, inflammation and cell survival. Activation of this pathway can lead to inflammatory diseases and even cancer, making it an important target for studying the effects of various compounds (KollarovaKollarova et al., 2018). A **Toll-like receptor (TLR) reporter gene assay** was used to measure the activation of TLR 2 and TLR4 after treatment with *Alternaria* toxins, which are key receptors in the innate immune system [STAMI (NO)]. These receptors recognize pathogen-associated molecular patterns. The **IL-2 Luc assay** according to OECD TG444A is based on a detailed review paper on immunosuppression that was developed by OECD and endorsed AOP 154 (Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response) (Komatsu et al., 2021; OECD 2022; OECD 2023d). In simple terms, reduced production of IL-2 by T helper cells negatively affects the proliferation and differentiation of B cells and lead to the suppression of the T cell–dependent antibody response (TDAR) [NIPH (NO)]. The establishment of this assay at NIPH will start in 2025 since it is difficult to purchase the cell line, although it is an OECD TG study. To study the impact of ENNs, BEA and *Alternaria* toxins on **intestinal barrier function**, the cytokine response in non-tumorigenic (HCEC-1CT) and tumorigenic (Caco-2) intestinal cells with and without IL1ß-stimulus will be assessed [UNIVIE (AT)]. In addition, several assays investigating the uptake, inflammation and tight junctions by using a 3D tissue model of the small intestine will be established [NVI (NO)].

Additional assays and *in silico* analysis

For most endpoints, cytotoxicity testing was a preliminary requirement and the partners performing cytotoxicity assessment in several cell types have shared the information in a database for general information.

***In silico* methods** have become valuable NAM tools in toxicity assessment, offering predictions for biotransformation and toxicity to help identify toxic compounds that needs to be further assessed. In the evaluation of the ENNs, two key computational approaches were used: Site of Metabolism scoring (SoM), which predicts biotransformation sites by analysing the compound's molecular structure and comparing it to known metabolic patterns, and Structure-Activity Relationship (SAR) analysis, which assesses the correlation between molecular structure and toxicity across 62 different endpoints, such as mutagenicity (*in vitro* and *in vivo*), non-genotoxicity (*in vitro* and *in vivo*), and hepatoxicity. Together these techniques provide valuable insights into the metabolic fate and toxicological profile of compounds, helping identify those that may cause adverse effects [IMR (NO)].

The **intrinsic clearance** refers to the ability of an organ, primarily the liver, to metabolize a substance independently of blood flow and protein binding. By using human, rat and mice hepatocytes, the intrinsic clearance of ENNs were determined [ANSES (FR)].

2.3. Results

In the following, the status of the work and the results are summarized in tables. Negative results are marked in green with a minus (-), while positive results are indicated in red with a plus (+). All results presented should be considered preliminary since all the experimental data are not yet available.

Genotoxicity and mutagenicity

Table 4 summarizes the status of the data from **Ames assay** for the *Alternaria* toxins. TeA did not induce mutagenic effects in TA98 and TA100 (+/- S9), TEN (up to 0.415 mg/plate) and ALT (up to 0.292 mg/plate) did not induce mutagenic effects in any of the tested strains (+/- S9). Therefore, TEN and ALT are considered to be non-mutagenic. In accordance with the criteria for a positive result (≥ 2-fold increase for S. Typhimurium (TA97a, TA98, TA100, TA102) and ≥ 3-fold increase for TA1535), AME (up to 0.272 mg/plate) induced frameshift mutations in the genome of Salmonella typhimurium strain (TA97a) without and with S9 metabolic activation (rat liver fractions, 10% S9) and did not induce a mutagenic effect in the strains TA98, TA100, TA102 and TA1535 (+/- S9) [NIB (SI)]. The studies for ENNs and BEA are still ongoing.

Table 4: Overview on the status of the Ames assay [NIB (SI)]

Substance		TA97a		TA98		TA100		TA102		TA1535	
		With S9	Without S9	With S9	Without S9	With S9	Without S9	With S9	Without S9	With S9	Without S9
Alternaria toxins	**AOH**	pending	pending	pending	pending	pending	pending	pending	pending	pending	pending
	AME	+ from 0,0086 mg/plate	+ from 0,0086 mg/plate	-	-	-	-	-	-	-	-
	ATX-I	ongoing	ongoing	ongoing	ongoing	ongoing	ongoing	ongoing	ongoing	ongoing	ongoing
	ALT	-	-	-	-	-	-	-	-	-	-
	TeA	ongoing	ongoing	-	-	-	-	ongoing	ongoing	ongoing	ongoing
	TEN	-	-	-	-	-	-	-	-	-	-

Green with a minus (-): negative results. red with a plus (+); positive results

tin A, A1, B, B1, BEA, ALT, and TEN showed negative results up to cytotoxic concentrations after 24h of exposure without S9, as well as after 3 hours of exposure with and without S9 in the **micronucleus assay in TK6 cells** (see Table 5). In contrast, ATX-I, AME, AOH, and TeA tested positive after 24h of exposure without S9, while AME was also positive after 3h of exposure with S9. Notably, TeA and ATX-I yielded negative results after 3h with S9, and TeA remained negative after 3h without S9 [Sciensano (BE)].

Table 5: Overview of the status from Micronucleus assay in TK6 cells [Sciensano (BE)]

Substance		24h without S9	3h with S9	3h without S9	Result summary
Enniatins	**ENN A**	-	-	-	-
	ENN A1	-	-	-	-
	ENN B	-	-	-	-
	ENN B1	-	-	-	-
	BEA	-	-	-	-
Alternaria toxins	**AOH**	+ from 2,50 - 4,00 µM (1)	pending	Not required	+
	AME	+ from 5,00 - 7,50 µM	+ from 25 µM	Not required	+
	ATX-I	+ from 0,05 - 0,25 µM	-	Not required	+
	ALT	- (2)	- (2)	- (2)	-
	TeA	+ (3)	-	-	+
	TEN	- (2)	- (2)	- (2)	-

Green with a minus (-): negative results. red with a plus (+); positive results

(1) Data from another batch as the one used in PARC

(2) Cytotoxic concentration not reached; max level based on SOP

(3) Repeat ongoing

The preliminary results of the **micronucleus assay in HepG2 cells** are summarized in Table 6. Regarding TeA, long exposure (48h) to 1, 10, 50, 75 and 100 µM revealed a significant increase in MN frequency in HepG2 cells at concentrations above 50 µM, as compared to non-exposed cells, while the short exposure (3h) caused a significant increase in MN above 75 µM. Regarding AME, 3 h exposure to 1, 5, 10, 20 and 30 µM did not result in a significant increase in the MN frequency. However, after 48 h exposure, there was a significant increase in MN frequency at concentrations of 20 µM and 30 µM, as compared to non-exposed cells. All experiments were performed without S9 [INSA(PT)].

Table 6: Preliminary results from Micronucleus assay in HepG2 cells [INSA (PT)]

Substance		24h without S9	3h with S9	3h without S9	Result summary
Alternaria toxins	**AOH**	pending	pending	pending	
	AME	+ from 20 µM	Not required	- from 5 - 30 µM	+
	ATX-I	ongoing	ongoing	ongoing	
	ALT	ongoing	ongoing	ongoing	
	TeA	+ from 50 µM	Not required	+ 75 µM (1)	+
	TEN	ongoing	ongoing	ongoing	

Green with a minus (-): negative results. red with a plus (+); positive results

(1) Highest concentration tested

Table 7 summarizes the preliminary results of the **SOS/umu test** with ENNs. ENN A, A1, B, B1 were negative in the SOS/umu test in absence and presence of S9 [UNAV(ES)].

Table 7: Preliminary results from SOS/umu [UNAV (ES)]

Substance		without S9	with S9	Result summary
Enniatins	**ENN A**	-	-	-
	ENN A1	-	-	-
	ENN B	-	-	-
	ENN B1	-	-	-
	BEA	ongoing	ongoing	
Alternaria toxins	**AOH**	ongoing	ongoing	
	AME	pending	pending	
	ATX-I	ongoing	ongoing	
	ALT	ongoing	ongoing	
	TeA	ongoing	ongoing	
	TEN	ongoing	ongoing	

Green with a minus (-): negative results. red with a plus (+); positive results

ENN A, A1, B, and B1 did not induce **γH2AX activation** at non-cytotoxic concentrations. However, at a toxic concentration of 10 µM, where cell viability (DAPI) was approximately 60–80%, ENN B, A, and B1 triggered γH2AX activation (see Table 8). No induction of γH2AX was observed for TEN, AME, ALT, and BEA. In contrast, ATX-I and TeA induced γH2AX activation even at non-cytotoxic concentrations [BfR (DE)].

Table 8: Status of γH2Ax data in HepaRG cells after 24h of incubation [BfR (DE)]

Substance		IC_{50} (µM)	γH2Ax		
			Result summary	Highest test concentration (µM)	Cell survival at highest test concentration (DAPI) relative to control
Enniatins	**ENN A**	60,54	(+), equivocal	10	75%
	ENN A1	18,31	-	10	99%
	ENN B	10,93	(+), equivocal	10	55%
	ENN B1	41,94	(+), equivocal	10	72%
	BEA	13,34	-	2	95%
Alternaria toxins	**AOH**	pending	pending	pending	pending
	AME	> 100	-	100	100%
	ATX-I	83,61	+	3	92%
	ALT	> 100	-	100	96%
	TeA	> 1000	(+), equivocal	1000	95%
	TEN	> 100	-	100	91%

Green with a minus (-): negative results. red with a plus (+); positive results

Results were interpreted as positive if fold change > 1,5 on two or more adjacent concentrations

Endocrine effects

In the **ER SSTA assay** (Table 9), ENNs were tested at concentrations ranging from 3 nM to 1 µM, with cytotoxicity remaining below 20% of the control. No agonist or antagonist effects were observed for any of the four ENNs. AME was tested at concentrations between 100 nM and 30 µM, TeA between 30 nM and 10 µM, and BEA between 3 nM and 1 µM. No antagonist effects were detected for these three mycotoxins. However, AME exhibited an agonist effect with an EC50 of 4.8–8.8 µM, whereas TeA acid and BEA showed no agonist effects. TEN and ALT were tested at concentrations from 100 nM to 30 µM, and neither exhibited agonist nor antagonist effects. ATX-I was tested within a range of 30 nM to 10 µM. No agonist effect was observed, but an antagonist effect was detected, with a LOAEL of 1 µM [INRS (FR)].

Table 9: Status of the results of the ER SSTA in hERα-HeLa9903 cells [INRS (FR)]

Substance		Dose range (µM)	Results	
			Agonist assay	Antagonist assay
Enniatins	**ENN A**	0 - 1 µM	-	-
	ENN A1	0 - 1 µM	-	-
	ENN B	0 - 1 µM	-	-
	ENN B1	0 - 1 µM	-	-
	BEA	0 - 1 µM	-	-
Alternaria toxins	**AOH**	pending	pending	pending
	AME	0 - 30 µM	+ TP_{50}: 4,8 - 8,8 µM	-
	ATX-I	0 - 10 µM	-	+ LOAEL 1 µM
	ALT	0 - 30 µM	-	-
	TeA	0 - 10 µM	-	-
	TEN	0 - 30 µM	-	-

Green with a minus (-): negative results. red with a plus (+); positive results

Regarding the **AR SSTA** assay, a sigmoidal concentration-response curve and an over 6.4-fold induction of AR activity using DHT was achieved. However, results with Mestanolone, so far, do not fit well in the sigmoidal curve (maximum response and thus plateau was usually achieved already at a concentration of 1.0 x 10−10 M). Standardisation with positive control Mestanolone and the reference compounds for the antagonist assay are currently ongoing. Experimentation with the test chemicals (Enniatins and *Alternaria* toxins) diluted in DMSO will follow [BPI (GR)].

The **steroidogenesis assay** (OECD TG 456) was performed to assess the endocrine potential of the mycotoxins. T According to the TG 456, cytotoxicity of ENNA, A1, B, B1 and BEA was evaluated as a preliminary step to exclude concentrations reducing vitality > 20%. The IC50 values are showed in Table 10. In two out of three biological replicates, ENNB and B1 showed an increase of E2 of 60% and 10%, respectively. The hormone assessment of the other mycotoxins are in progress [ISS (IT), BfR (DE)].

Table 10: Preliminary results of the Steroidogenesis Assay [ISS (IT) and BfR (DE)]

Substance		IC_{50} (µM)	Steroidogenesis		
			Max concentration tested (µM)	E2	T
Enn iati	**ENN A**	1,06 ± 0,08		ongoing	ongoing

	ENN A1	3,48 ± 0,50		ongoing	ongoing
	ENN B	> 10	1	↑ 60% increase (1)	ongoing
	ENN B1	7,14 ± 0,30	1	↑ 10% increase (1)	ongoing
	BEA	3,08 ± 0,50		ongoing	ongoing
***Alternaria* toxins**	**AOH**	pending	pending	pending	pending
	AME	ongoing	ongoing	ongoing	ongoing
	ATX-I	ongoing	ongoing	ongoing	ongoing
	ALT	ongoing	ongoing	ongoing	ongoing
	TeA	ongoing	ongoing	ongoing	ongoing
	TEN	ongoing	ongoing	ongoing	ongoing

(1) Preliminary results of two out of three biological replicas; statistical analysis was not performed

An **E-screen assay** was performed to evaluate the endocrine potential of the *Alternaria* toxins. The preliminary results are summarized in Table 11.

Table 11: Preliminary results of the E-Screen assay [BPI (GR)]

Reference compound		**Results**
17-β-estradiol (positive control)		Estradiol treatment (10^{-10} M - 10^{-8} M) has resulted in a statistically significant fold increase in cell proliferation. The optimal concentration was set at 10^{-9} M, which led to an increase in the range of 2-2,5 compared to control. This is in accordance with scientific literature using MCF-7 cells from ATCC and estradiol treatment (fold change of 1,5-3). The cell system is considered responsive to ER-mediated proliferation agonists.
Fulvestrant (ICI 182,780) (inhibition control)		Treatment of cells with the pure antagonist ICI 182,780 led to a statistically significant decrease in cell proliferation compared to control. Co-incubation of estradiol with ICI 182,780 (10^{-7} M ICI 182,780 and 10^{-9} M 17-β-estradiol) also significantly decreased the magnitude of E2-induced cell proliferation as expected.
Mycotoxins		
***Alternaria* toxins**	**AOH**	Ongoing. A range of concentrations per compound will be tested (alone or in combination with ICI 182,780) to assess agonists and antagonists of the ER-mediated proliferation. We are currently working with Tenuazonic acid.
	AME	
	ATX-I	
	ALT	
	TeA	
	TEN	

Immunotoxic effects

In the **NF-κB reporter gene assay**, AOH and ATX-I suppressed the LPS-induced activation of the NF-kB pathway in the absence of S9 (Table 12). TeA did not interact with the pathway (up to 250 μM). Suppression of the pathway by AME was observed only at cytotoxic concentrations, thus no clear indication of a real interaction with the pathway could be concluded. Also, suppression of the pathway by ENNA, ENNA1, ENNB,

ENNB1 and BEA was observed only at cytotoxic concentrations, thus no indication of a clear interaction with the pathway was observed [UNIVIE (AT)].

Table 12: Status of the results of the NF-κB reporter gene assay and CellTiter Blue assay in the presence of LPS stimulation in the absence of S9 [UNIVIE (AT)]

Substance		Cell viability (%) *		Luciferase activity (%) *			Summary results
		Lowesst active conc.	Highest active conc.	Active concentration range (µM)	Lowest active conc.	Highest active conc.	
Enniatins	**ENN A**	≈ 60*	≈ 50*	2,5 - 5,0	≈ 70*	≈ 40*	No clear immunosuppressive effects (concurrent cytotoxicity)
	ENN A1	≈ 80	≈ 70*	2,5 - 5,0	≈ 80*	≈ 50*	
	ENN B	≈ 70*	≈ 65*	2,5 - 5,0	≈ 80	≈ 75	
	ENN B1	≈ 65*	≈ 60*	2,5 - 5,0	≈ 70*	≈ 60*	
	BEA		≈ 60	5		≈ 80*	
Alternaria toxins	**AOH**	≈ 99	≈ 85#	1 - 20	≈ 80	≈ 18	+
	AME	≈ 76#	≈ 77#	5 - 20	≈ 84	≈ 62	No clear immunosuppressive effects (concurrent cytotoxicity)
	ATX-I	≈ 90	≈ 98	1 - 20	≈ 95	≈ 56	+
	ALT	ongoing	ongoing	ongoing	ongoing	ongoing	
	TeA	-	-	-	-	-	- up to 250 µM
	TEN	ongoing	ongoing	ongoing	ongoing	ongoing	

* Results are expressed in percentage compared to the control. Treatment conditions: 20h, with LPS stimulation
# Significantly different compared to the control (p<0,05)
§ Experiments in the presence of S9 fractions are going

Regarding the TLR reporter gene assay, results are summarized in Table 13. AOH and AME show some reduction of LPS-induced **TLR-NFκB signal**, while ATX-I increased the TLR-NFκB signal in the absence of LPS. ALT, TeA and TEN did not significantly impact the TLR-NFκB signal alone or in combination with LPS [STAMI (NO)].

Table 13: Status of the results from TLR reporter gene assay [STAMI (NO)]

Substance		Highest test concentration (µM) *	TLR2		TLR4	
			Induce TLR-NFk-β pathway	Inhibit LPS activated TLR-NFk-β pathway	Induce TLR-NFk-β pathway	Inhibit LPS activated TLR-NFk-β pathway
Alternaria toxins	**AOH**	30	-	+	-	+
	AME	10	-	+	-	+
	ATX-I	20	+	-	+	-
	ALT	10	-	-	-	-
	TeA	100	-	-	-	-
	TEN	10	-	-	-	-

Green with a minus (-): negative results. red with a plus (+); positive results

* No cytotoxicity was observed at the dose ranges tested (alamarBlue)

Additional assays and *in silico* analysis

The *in silico* analysis, utilizing **Site of Metabolism Scoring,** indicated that ENNs and BEA are primarily metabolised through the Phase I CYP450 system (Table 14). While the Meteor software predicted only hydroxylations and demethylations, additional theoretical metabolites are described in the literature. The Structure-Activity Relationship (SAR) assessment suggested that BEA has a higher toxic potential compared to the ENNs [IMR (NO)].

Table 14: In silico predictions for biotransformation and toxicity based on Site of Metabolism Scoring and Structure-Activity Relationship (SAR) analysis [IMR (NO)]

Substance		Biotransformation	Predicted biotransformation reactions that can produce metabolites	Toxicity
Enniatins	**ENN A**	Phase 1, CYP450	Oxidative N-demethylation and hydroxylation of terminal methyl	Potential hepatoxic compounds after hydrolysis and ring-opening
	ENN A1	Phase 1, CYP450	Oxidative N-demethylation and hydroxylation of terminal methyl	-
	ENN B	Phase 1, CYP450	Oxidative N-demethylation	-
	ENN B1	Phase 1, CYP450	Oxidative N-demethylation and hydroxylation of terminal methyl	-
	BEA	Phase 1, CYP450	Oxidative N-demethylation and para hydroxylation at monosubstituted benzene compounds	Several metabolites with alert for hepatoxicity since the benzene rings can be transformed to alkylphenls. Alkylphenols can cause chromosome aberration.

The predicted intrinsic clearance on human, rat and mice hepatocytes were summarized in Table 15 [ANSES (FR)].

Table 15: Intrinsic Clearance on human and rat and mice hepatocytes [ANSES (FR)]

Substance		Parameters	Unit	Human	Rat	Mice
Enniatins	**ENN A**	Half-life	H	ongoing	ongoing	n.d.
		Constant of elimination (Ke)	/h	ongoing	ongoing	n.d.
	ENN A1	Half-life	H	ongoing	ongoing	n.d.
		Constant of elimination (Ke)	/h	ongoing	ongoing	n.d.
	ENN B	Half-life	H	1,02	0,48	0,29
		Constant of elimination (Ke)	/h	0,68	1,43	2,41
	ENN B1	Half-life	H	1,07	0,55	n.d.
		Constant of elimination (Ke)	/h	0,65	1,27	n.d.
Alternaria toxins	**TeA**	Half-life	H	ongoing	ongoing	n.d.
		Constant of elimination (Ke)	/h	ongoing	ongoing	n.d.
	TEN	Half-life	H	ongoing	ongoing	n.d.
		Constant of elimination (Ke)	/h	ongoing	ongoing	n.d.

n.d. not determined

3. Environmental effects of natural aquatic toxins

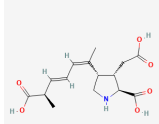
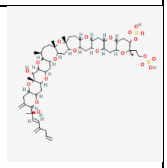
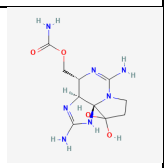
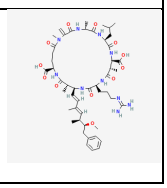
This project contributes to the effect assessment of natural toxins in aquatic ecosystems using standard OECD testing procedures that can be directly linked to risk assessment approaches. Particularly, the project also provides comprehensive information on the ecotoxicity of natural toxins in mixtures using standard OECD tests and therefore contributes to a more realistic risk assessment of natural toxins and their impact on achieving good environmental status (i.e., water framework directive and marine framework directive).

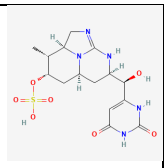
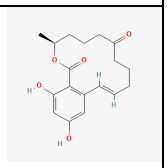
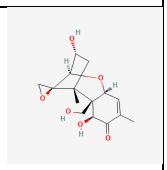
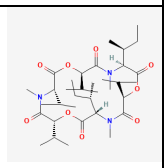
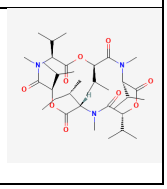
3.1. Substances

Procurement of substances:

Substances were either commercially acquired or collected through a harmful algae producer obtained from a certified culture collection (Table 16). The producer was *Microcystis aeruginosa* (PCC 7806). In experiments where this producer was used, it was cultured by our partner, UGent (BE), to ensure consistency across all partners working with the same organism and minimize batch to batch variation. For experiments involving *Chloarella vulgaris,* producer strains that could grow in the same culture medium were selected: *Microcystis aeruginosa* (LEGE 91094) and *Aphanizomenon ovalisporum* (LEGE X-001). As each partner tested different toxins in various test systems, a centralized purchase of these strains was not necessary. Partner UAVR (PT) also added additional reference chemicals to assess the effects of binary mixtures. The chemical substances used in this study included cadmium chloride anhydrous ($CdCl_2$, 99% purity, FLUKA, Sigma-Aldrich), and TBA ($C_9H_{16}ClN_5$, molecular weight: 229,7 Daltons), tested as a commercial formulation (SAPEC, containing 500 g/L active ingredient TBA).

Table 16: List of natural aquatic toxins investigated in P5.1.2.a, visual structures are obtained from PubChem

	Substance	Abbreviation	CAS no.	Molecular weight (g/mol)	Structure	Visual structure
Cyanobacterial toxins	Domoic acid	**DA**	14277-97-5	311,3	$C_{15}H_{21}NO_6$	
	Yessotoxin	**YTX**	112514-54-2	114344,0	$C_{55}H_{82}O_{21}S_2$	
	Saxitoxin	**STX**	35523-89-8	299,3	$C_{10}H_{17}N_7O_4$	
	Microcystin-LR	**MC-LR**	101043-37-2	995,2	$C_{49}H_{74}N_{10}O_{12}$	

	Cylindrospermopsin	**CYL**	143545-90-8	415,4	$C_{15}H_{21}N_5O_7S$	
Mycotoxins	Zearalenone	**ZEN**	17924-92-4	318,4	$C_{18}H_{22}O_5$	
	Deoxynivalenol	**DON**	51481-10-8	296,3	$C_{15}H_{20}O_6$	
	Enniatin A1	**ENN A1**	4530-21-6	667,9	$C_{34}H_{59}N_3O_9$	
	Enniatin B1	**ENN B1**	19914-20-6	653,8	$C_{35}H_{61}N_3O_9$	

3.2. Target species, endpoints and methods

In this section a summary of the organisms, different endpoints and methods used is presented. More detailed description of the methods can be found in the Annex of this deliverable.

Following the REACH regulation, model algae and invertebrate species are used to assess effects of natural toxins on life history endpoints of these organisms. The selected model species include *Chlorella vulgaris* LEGE Z-001, *Lymnea stagnalis, Daphnia magna, Nitocra spinipes* and *Acartia tonsa.* These are standard model species across freshwater, estuarine and marine environments in current REACH regulations and environmental risk assessment frameworks. Table 17 summarizes the test guidelines followed by the different partners on the target species selected.

Table 17: Test guidelines followed on the selected model species

Test guideline	Organism
OECD 211: Daphnia magna reproduction test	*Daphnia magna*
ISO14669: Determination of acute lethal toxicity to marine copepods	*Nitocra spinipes* *Acartia tonsa*
ISO18220: Larval development test with the harpacticoid copepod Nitocra spinipes	*Nitocra spinipes*
OECD 243: Lymnaea stagnalis Reproduction Test	*Lymnea stagnalis*
OECD 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test	*Chlorella vulgaris*

Table 18 presents an overview of the different tests performed to assess single toxins. Acute and chronic toxicity experiments were executed and the following endpoints were evaluated: mortality, swimming speed, development and reproduction.

Table 18: Overview of all conducted tests with single toxins

Substance	Organism	Lifestage	Exposure Time	Endpoint
DA	*N. spinipes*	Adult	Acute	Mortality
		Naupli	Acute	Mortality
			Chronic	Development
			Chronic	Reproduction
YTX	*N. spinipes*	Adult	Acute	Mortality
		Naupli	Acute	Mortality
			Chronic	Development
			Chronic	Reproduction
STX	*N. spinipes*	Naupli	Chronic	Development
MC-LR	*N. spinipes*	Naupli	Chronic	Reproduction
		Adult	Acute	Swimming speed
			Acute	Mortality
	A. tonsa	Adult	Acute	Swimming speed
			Acute	Mortality
	L. stagnalis	Naupli	Chronic	Reproduction
ZEN	*L. stagnalis*	Naupli	Chronic	Reproduction

Additional mixtures studies are currently ongoing and Table 19 collects the mixtures that have been tested. Chronic exposure experiments with *Lymnaea stagnalis* evaluated effects of microcystin, 0.5 to 50 µg/L, Zearalenon, 0.030 to 0.50 mg/L, while mixture studies on Deoxynivalenol (Don), Zearalenone (Zen), Enniatin A1 (EnnA1) and Enniatin B1 (EnnB1) based on environmental relevant levels is currently ongoing for this organism. The lowest mixture was 0.1, 0.05, 0.03 and 0.01 µg/L of Don, Zen, EnnA1 and EnnB1, respectively. The remaining tested concentrations consisted of the same relative ratios of the four mycotoxins but concentrated 10, and 100 times compared to the lower mixture. Besides *L. stagnalis*, the combined toxicity of cyanotoxins and anthropogenic contaminants on the growth of *Chlorella vulgaris*, focusing on both single and binary mixture exposures, has been also analysed. The test substances included cadmium chloride ($CdCl_2$), terbuthylazine (TBA), microcystin-LR (MC-LR), and cylindrospermopsin (CYN), due to their environmental relevance and potential interactive effects. A fixed ray experimental design was used for all experiments. For MC-LR combination with TBA and Cd, the experimental design consisted of single exposures to 7 concentrations of MC-LR and 5 concentrations of TBA or Cd and 25 combinations of MC-LR and TBA or MC-LR and Cd. The nominal concentrations for MC-LR single exposures ranged from 0.5 to 80 mg.L-1 and for combined exposures, from 0.5 to 20 mg.L-1. TBA and Cd nominal concentrations ranged from 0.03 to 0.48 and 0.05 to 5 mg.L-1 in single and combined exposures. For CYN combination with TBA and Cd, the experimental design consisted of single exposures to 4 concentrations of CYN and 5 concentrations of TBA and Cd and 25 combinations of substances for each case. The nominal concentrations for CYN single exposures ranged from 10 to 80 mg.L-1 and for combined exposures, from 0.5 to 20 mg.L-1. TBA and Cd nominal concentrations ranged from 0.03 to 0.48 and 0.05 to 5 mg.L-1 in single and combined exposures. Interactions between chemicals in binary mixtures were analyzed to determine whether they exhibited synergistic, additive, or antagonistic effects. For experiments with copepods, we used bloom-

relevant cell densities of *M. aeruginosa* and *A. minutum* in our mixture experiments. As these two species differ in cell size, we standardized biomass based on carbon content. The average carbon content per cell was 1.71 ± 0.48 pg cell-1 for M. aeruginosa and 469.70 ± 30.59 pg cell-1 for A. minutum. Accordingly, four cell densities of M. aeruginosa were selected, ranging from 0.25 to 2 × 106 cells mL-1 with a two-fold dilution between levels. To achieve an equivalent biomass (carbon content) ratio, four corresponding cell densities of A. minutum were chosen, ranging from 1000 to 8000 cells mL-1, also using a two-fold dilution series. *Microcystis aeruginosa* blooms have been reported to occur in estuaries by previous studies, in densities ranging from 0.029 x 106 to 2.7 x 106 cells mL-1 (Bormans et al., 2020; Taş et al., 2006). Alexandrium minutum blooms have been observed at densities between 104 to 106 cells L-1 (Bravo et al., 2008; Cosgrove et al., 2014). Nitocra spinipes is an estuarine copepod species with a broad salinity tolerance (Svetlichny and Hubareva, 2014; Wulff, 1972). For Daphnia, a previous acute toxicity test conducted by the University of Birmingham reported a 72 h EC50 with immobility at 9.5 × 106 cells mL-1. To facilitate a two-fold dilution series, we selected 1 × 107 cells mL-1 as the highest exposure concentration of *M. aeruginosa*, resulting in four exposure levels. The same cell densities of *A. minutum* (1000 to 8000 cells mL-1) were maintained for consistency.

Table 19: Summary of mixtures tested

Organism	Test guideline	Mixture	Status
Lymnea stagnalis	OECD 243	MC-LR + ZEN	done
		MC-LR + DON	ongoing
		MC-LR + ENN A1	ongoing
		MC-LR + ENN B1	ongoing
Chlorella vulgaris	OECD 201	MC-LR + TBA	ongoing
		MC-LR + $CdCl_2$	ongoing
		CYN + TBA	ongoing
		CYN + $CdCl_2$	ongoing
Daphnia magna	OECD 211	Saxitoxin producer + microcystin LR producer	ongoing
Nitocra spinipes	ISO14669/ ISO18220	Saxitoxin producer + microcystin LR producer	ongoing

3.3. Results

Results from UGent and UAVR are a summary from results of published work (see scientific achievements). All results are based on nominal concentrations. Firstly, we determined acute effect concentrations for *N. spinipes* (Table 20). Secondly, we observed significant impacts of yessotoxin and saxitoxin on the brood size of *N. spinipes* (Table 21). Lastly, elevated temperatures markedly intensify the reproductive toxicity of algal toxins including domoic acid, yessotoxin and saxitoxin (Table 21). Notably, the concentrations of the four algal toxins in natural seawater, in the absence of blooms, are currently below the effective concentrations identified in this study, suggesting a low risk of exposure for marine copepods. Furthermore, exposure to MC-LR producing cyanobacteria resulted in decreased swimming speed, increased inactivity, and higher mortality in *A. tonsa*, compared to the non-toxic algae (Table 22, 23). No effects were observed for *N. Spinipes* (Table 22, 23). For *L. stagnalis,* all results are presented as nominal concentrations. No effects at tested concentrations were observed for MC-LR and ZEN (Table 24).

Table 20: Acute toxicity tests with N. spinipes to determine the 50% Lethal concentration [UGent (BE)] from Liu et al. 2024

Substance	Temperature	Adults		Nauplii	
		Concentration Range	LC50	Concentration Range	LC50

DA	15°C	0 - 1000 µg L^{-1}	25,97 ± 11,96 µg L^{-1}	0 - 600 µg L^{-1}	97,24 ± 6,45 µg L^{-1} *
YTX	15°C	0 - 150 µg L^{-1}	ND	0 - 120 µg L^{-1}	ND
DA	20°C	0 - 1000 µg L^{-1}	17,15 ± 3,34 µg L^{-1}	0 - 600 µg L^{-1}	81,99 ± 3,89 µg L^{-1}
YTX	20°C	0 - 150 µg L^{-1}	ND	0 - 120 µg L^{-1}	ND
DA	25°C	0 - 1000 µg L^{-1}	8,79 ± 1,93 µg L^{-1}	0 - 600 µg L^{-1}	57,26 ± 6,82 µg L^{-1} *
YTX	25°C	0 - 150 µg L^{-1}	ND	0 - 120 µg L^{-1}	ND

* Tests were conducted at 18/22°C instead of 15/25°C due to mortality of the Nauplii at 15/25°C.
ND: LC50 could not be determined, as even in the highest concentrations tested mortality did not reach 50%

Table 21: Chronic toxicity tests with N. spinipes to determine effects on developmental and reproductive toxicity [UGent (BE)] from Liu et al. 2024

Substance	Temperature	Developmental toxicity		Reproductive Toxicity	
		Concentration Range	LOEC	Concentration Range	LOEC
DA	18°C	0 - 10 µg L^{-1}	No Effects	0 - 10 µg L^{-1}	No Effects
YTX	18°C	0 - 5 µg L^{-1}	No Effects	0 - 5 µg L^{-1}	No Effects
STX	18°C	0 - 20 µg L^{-1}	5 µg L^{-1}	0 - 20 µg L^{-1}	10 µg L^{-1}
MC-LR	18°C	0 - 200 µg L^{-1}	100 µg L^{-1}	0 - 200 µg L^{-1}	No Effects
DA	20°C	0 - 10 µg L^{-1}	No Effects	0 - 10 µg L^{-1}	No Effects
YTX	20°C	0 - 5 µg L^{-1}	No Effects	0 - 5 µg L^{-1}	5 µg L^{-1}
STX	20°C	0 - 20 µg L^{-1}	No Effects	0 - 20 µg L^{-1}	10 µg L^{-1}
MC-LR	20°C	0 - 200 µg L^{-1}	No Effects	0 - 200 µg L^{-1}	No Effects
DA	22°C	0 - 10 µg L^{-1}	No Effects	0 - 10 µg L^{-1}	No Effects
YTX	22°C	0 - 5 µg L^{-1}	No Effects	0 - 5 µg L^{-1}	5 µg L^{-1}
STX	22°C	0 - 20 µg L^{-1}	No Effects	0 - 20 µg L^{-1}	10 µg L^{-1}
MC-LR	22°C	0 - 200 µg L^{-1}	No Effects	0 - 200 µg L^{-1}	200 µg L^{-1}

The table reports the lowest observed effect concentrations

Table 22: Acute toxicity tests with N. spinipes and Acartia tonsa to determine effects of MC-LR producer on swimming behaviour and mortality under a combination of increased temperature and salinity at a fixed ratio of MC-LR producer strain [UGent (BE)] from Liu et al. 2025.

	Variable	*N. spinipes*		*A. Tonsa*	
		% of Inactivity	Average speed	% of Inactivity	Average speed
Temperature x MC-LR producer	20°C	No Effects	No Effects	+	+
	24°C	No Effects	No Effects	+	+
	26°C	No Effects	No Effects	+	+
	28°C	No Effects	No Effects	+	+
Salinity x	8 ppt	No Effects	No Effects	+	+

MC-LR Producer	9,5 ppt	No Effects	No Effects	+	+
	12,5 ppt	No Effects	No Effects	+	+

+ treatments that significantly differed from control

Table 23: Acute toxicity tests with N. spinipes and Acartia tonsa to determine effects of MC-LR producer on swimming behaviour and mortality under a combination of increased temperature and salinity at a fixed ratio of MC-LR producer strain. The table reports the lowest observed effect concentrations. A Plus sign indicates treatments that significantly differed from control [UGent (BE)] from Liu et al. 2025.

Salinity	Temperature	*N. spinipes*	*A. Tonsa*
		Mortality	Mortality
8ppt	20°C	No Effects	+ (>50%)
	24°C	No Effects	+ (>50%)
	26°C	No Effects	+ (>50%)
	28°C	No Effects	+ (>50%)
9,6ppt	24°C	No Effects	+ (>50%)
	26°C	No Effects	+ (>50%)
	28°C	No Effects	+ (>75%)
12,8 ppt	26°C	No Effects	+ (>50%)
	28°C	No Effects	+ (>50%)

Table 24: Results of the Lymnaea stagnalis reproduction test [SLU (SE)]

Organism	Substance	Concentration range	Reproduction
Lymnaea stagnalis	**MC-LR**	0,5 - 50 µg L^{-1}	No Effects
	ZEN	0,03 – 0,50 mg L^{-1}	No Effects

In the single expore experiments for *C. vulgaris*, TBA was the most toxic compound, approximately five times more toxic than Cd after four days, while Cd was 28 times more toxic than CYN (Table 25). By day seven, the gap between TBA and Cd narrowed, but CYN remained significantly less toxic. MC-LR was the least toxic, never reaching 50% growth inhibition.

Table 25: NOEC, LOEC and EC50 values (with corresponding standard errors), in mg.L-1, for the effect of selected chemical substances on the growth rate of Chlorella vulgaris exposed for 4 and 7 days in Z8 medium. NOEC is the no observed effects concentration, LOEC the lowest observed effect concentration and EC50 the median effect concentration.

Substances	NOEC		LOEC		EC_{50} (± SE)	
	Day 4	Day 7	Day 4	Day 7	Day 4	Day 7
Microcystin-LR (MC-LR)	> 37.3	23.9	> 37.3	37.3	> 37.30	> 37.30
	40.4	40.4	78.9	78.9	> 78.9	> 78.9

	19.6	40.4	40.4	78.9	> 78.9	> 78.9
Cylindrospermopsin (CYN)	4.4	> 16.7	8.5	> 16.7	> 16.70 (n.d.)	> 16.70 (n.d.)
	17.3	9.6	38.7	17.3	49.21 (± 3.29)	47.67 (± 2.98)
	17.3	9.6	38.7	17.3	56.34 (± 2.73)	51.26 (± 1.72)

Binary mixtures showed varying interactions (Figure 2, 3). MC-LR and TBA exhibited antagonism at low doses, with potential synergism at higher concentrations (Figure 2). A similar pattern appeared for MC-LR and Cd, where Cd dominance led to antagonism, but MC-LR dominance induced synergism (Figure 2). CYN and TBA were mostly antagonistic, with some signs of synergism at high levels, while CYN and Cd consistently showed antagonism (Figure 3). The TBA and Cd mixture remained antagonistic throughout (Figure 3). Overall, TBA posed the highest risk to *C. vulgaris*, while MC-LR had minimal impact. Most interactions were antagonistic, with occasional synergism at specific concentrations, emphasizing the need to account for mixture effects in ecological risk assessments.

Figure 2: Concentration-response relationship for the binary mixtures of microcystin-LR and terbuthylazine and microcystin-LR and cadmium tested on the growth rate of C. vulgaris after 4 and 7 days of exposure (2D isobolic surface). A, B and D show a dose level deviation from the CA model; C shows an antagonistic pattern after CA model fit. Concentrations of microcystin-LR reported as effective values and concentrations of terbuthylazine and cadmium reported as nominal concentrations [UAVR (PT)] From Loureiro et al. (submitted).

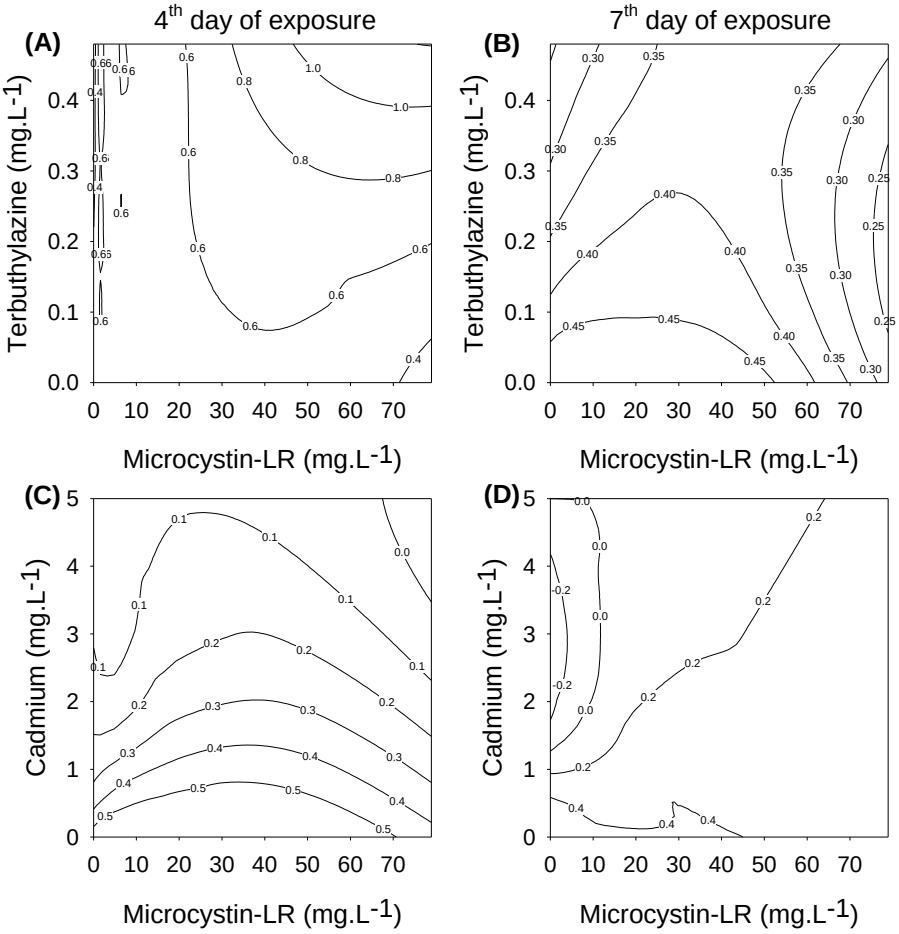
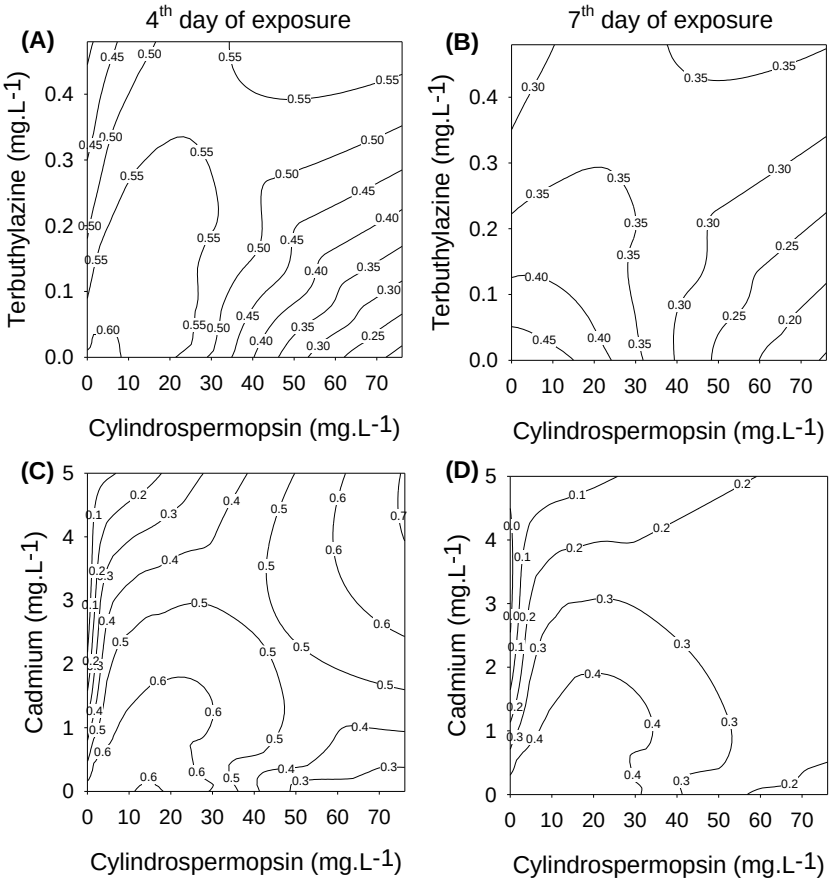


Figure 3: Concentration-response relationship for the binary mixtures of cylindrospermopsin and terbuthylazine and cylindrospermopsin and cadmium tested on the growth rate of C. vulgaris after 4 and 7 days of exposure (2D isobolic surface). A shows a dose ratio deviation from the CA model; B shows a dose level deviation from the CA model; C and D show an antagonistic pattern after CA model fit. Concentrations of cylindrospermopsin reported as effective values and

concentrations of terbuthylazine and cadmium reported as nominal concentrations [UAVR (PT)]. From Loureiro et al. (submitted).



4. Conclusions

The studied ENNs including BEA generated negative results in the micronucleus assay in TK6 cells, indicating that these toxins do not produce chromosomal damage under the conditions tested. However, the results of the γH2Ax assay, which reflects events of repair of DNA double strand breaks, are less clear for ENN A, B and B1 tested in HepaRG cells. In addition, negative results were obtained in the SOS/umu test for ENN A, A1, B and B1 (BEA still pending). No endocrine disruptive effects were seen for the ENNs in the ER SSTA assay in hERα-HeLa9903 cells. The steroidogenesis assay is still ongoing. With respect to immunotoxic effects, a decrease of LPS-induced NF-κB activation in THP-1 monocytes was only observed in cytotoxic concentrations. Studies in intestinal cells (cancer vs. non-transformed cells) are still ongoing. In addition, for ENNs *in silico* predictions for biotransformation and toxicity were calculated. Detection of the intrinsic clearance in human, rat and mice hepatocytes has been completed for ENN B and B1 and is ongoing for ENN A and A1.

ALT and TEN were found negative in the AMES test. Preliminary data for the *Alternaria* toxins AOH, AME, ATX-I and TeA were positive in the micronucleus test. However, the data set for both selected test systems is not complete yet. ATX-I was also positive in the γH2Ax assay. With respect to endocrine disruptive effects, AME was positive in the agonistic assay (ER SSTA) in hERα-HeLa9903 cells (AOH still pending). No agonistic activity was found for ATX-I, ALT, TeA and TEN. However, ATX-I showed antagonistic effects (LOAEL 1µM). The steroidogenesis assay and the E-screen assay are currently ongoing. So far, immunotoxic effects were studied in two different reporter gene systems. In HEK293TLR-NFkB reporter gene cells, AOH and AME inhibited LPS-activation of the TLR2 and TLR4 signal, whereas inducing effects were observed for ATX-I. The results for AOH and AME are in accordance with results in THP1 monocytes. AOH and AME were found to suppress the LPS-induced activation of NF-κB. But for AME suppressive effects co-occurred with cytotoxicity, thus the results are less clear. In this test system, no

stimulatory activity was observed for ATX-I. In THP-1 monocytes, ATX-I suppresses LPS-induced activation of NF-κB, arguing for immunosuppressive activity. In both test systems, TeA was not active. On the level of the Toll-like receptor, ALT and TEN were clearly inactive in the TLR reporter gene system. The studies for ALT and TEN in THP-1 monocytes are ongoing. Further studies, currently in progress, address immunotoxic effects on intestinal cells as the first target site after oral exposure, comparing cancer cells to non-transformed cells.

Taken together, for both classes of emerging mycotoxins crucial data gaps for hazard characterization are currently systematically addressed to improve future hazard assessment.

For natural aquatic toxins, we filled crucial data gaps within the environment. Firstly, we observed significant impacts of yessotoxin and saxitoxin on the brood size of *N. spinipes*. Second, elevated temperatures markedly intensify the reproductive toxicity of algal toxins including domoic acid, yessotoxin and saxitoxin. Notably, the concentrations of the four algal toxins in natural seawater, in the absence of blooms, are currently below the effective concentrations identified in this study, suggesting a low risk of exposure for marine copepods. Furthermore, exposure to MC-LR producing cyanobacteria resulted in decreased swimming speed, increased inactivity, and higher mortality in *A. tonsa*, compared to the non-toxic algae. No effects were observed for *N. Spinipes.* Simiarly, for *L. stagnalis,* no effects at tested concentrations were observed for MC-LR and ZEN. For *C. vulgaris*, the natural toxin CYN was significantly less toxic than chemical pollutants, MC-LR was the least toxic, never reaching 50% growth inhibition. Binary mixtures showed varying interactions, implying that mixture effects with aquatic toxins cannot be excluded. Most interactions were antagonistic, with occasional synergism at specific concentrations, emphasizing the need to account for mixture effects in ecological risk assessments.

Part II: BPA Alternatives: Human Health & Environment

1. Introduction

Concern has been raised regarding the potential health risks associated with environmental and human exposure to bisphenol A (BPA), a synthetic chemical that is widely used in the manufacturing of epoxy resins and polycarbonate plastic. EFSA has recently reduced the TDI and the EU commission has banned the use of BPA in food contact materials.

BPA was added to the candidate list of Substances of Very High Concern in the EU due to endocrine disruptor properties and reprotoxic effects, with consequent ban or restricted industrial use. Industry is exploring BPA alternatives, some of these BPA alternatives are already used by industry. Data gaps for hazard and metabolism do exist for some of these BPA alternatives. These gaps of novel BPA alternatives need to be closed in order to protect human health and the environment and to avoid regrettable substitutions.

The project **P5.1.1.b** focuses on the toxicity of BPA alternatives in **human health**, whereas the project **P5.2.1.b** addresses their impact on the **environment**. Therefore, we are sharing the data of both projects accordingly.

2. Substances

Bisphenol A alternative substances (Table 26) were selected and prioritized with all relevant stakeholders during a workshop organized in June 2022 [Aiello et al. 2023]. In addition, thorough literature reviews were performed to identify the knowledge and data gaps for the selected BPA alternatives regarding both human health and the ecosystem. In Mhaouty-Kodja et al. (2024) "A critical review to identify data gaps and improve risk assessment of bisphenol A alternatives for human health" data gaps regarding adverse human health outcomes were described as a basis for this project. Adamovsky et al. (2024) in "Exploring BPA alternatives – Environmental levels and toxicity review" emphasizes the importance of considering mixtures, realistic environmental concentrations, and the long-term consequences of exposure to BPA alternatives on biota and ecosystems.

The project dedicated to Environmental Health goes beyond the prioritized BPA alternatives and included a second and third group of substances.

For reducing interlaboratory differences in test substance characterisation, a centralized order was established during PARC Year 1 by LIH (LU) with Chiron (order placed the 10/2023). The purity of the prioritized substances was verified by **HPLC-UV** and assessed by **H-NMR** [INRAE (FR)]. These analyses confirmed that all prioritized molecules have a purity of more than 99%.

Table 26: Prioritized BPA Alternatives for both human and environmental hazards

Substance	Abbreviation	CAS no.	Molecular weight (g/mol)	Structure
Group 1				
Bisphenol A	**BPA**	80-05-7	228,29	$C_{15}H_{16}O_2$
Bisphenol Z	**BPZ**	843-55-0	268,35	$C_{18}H_{20}O_2$
Bisphenol E	**BPE**	2081-08-5	214,26	$C_{14}H_{14}O_2$
Bisphenol S-MAE	**BPS-MAE**	97042-18-7	290,34	$C_{19}H_{16}O_4S$
Pergafast 201		232938-43-1	460,52	$C_{21}H_{20}N_2O_6S_2$

Bisphenol P	**BPP**	2167-51-3	346,46	$C_{24}H_{26}O_2$
Bisphenol AP	**BPAP**	1571-75-1	290,36	$C_{20}H_{18}O_2$
Tetrachlorobisphenol A	**TCBPA**	79-95-8	366,07	$C_{15}H_{12}Cl_4O_2$
Tetrabromobisphenol A	**TBBPA**	79-94-7	543,88	$C_{15}H_{12}Br_4O_2$
Group 2 - Environment				
Bisphenol P Hydroquinone	**BPPH**	24038-68-4	380,48	$C_{27}H_{24}O_2$
Bisphenol S-MPE	**BPS-MPE**	63134-33-8	340,40	$C_{19}H_{16}O_4S$
Group 3 - Environment. Substances that are frequently identified in environmental compartments (e.g. surface water, soil, sediment)				
Bisphenol B	**BPB**	77-40-7	242,31	$C_{16}H_{18}O_2$
Bisphenol F	**BPF**	620-92-8	200,23	$C_{13}H_{12}O_2$
Bisphenol S	**BPS**	201-250-5	250,27	$C_{12}H_{10}O_4S$
Bisphenol AF	**BPAF**	216-036-7	336,23	$C_{15}H_{10}F_6O_2$

A harmonised SOP for the preparation of stock solutions and dilutions of BPA alternatives was first developed and shared among WP5 partners, taking into account preliminary solubility and stability data. This SOP has been continuously updated as new information becomes available. Solubility and bioavailability studies are currently ongoing for seven prioritized BPA alternatives across several laboratories [INRAE (FR), ISS (IT), UMIL (IT), ISCIII (ES), LIH (LU), MUI (AT)]. Notably, Pergafast 201 was not tested by MUI and UMIL. The SOP now incorporates both finalized solubility data and ongoing bioavailability results in various cell culture media [INSA (PT), ISS (IT), UMIL (IT), TTL (FI), ULFFA (SI), ISCIII (ES), LIH (LU), NILU (NO), MUI (AT)]For all BPA alternatives, cytotoxicity studies were carried out in HepaRG cells to determine the highest non-cytotoxic concentration (action performed mostly by [INRAE (FR)]). Partners performing cytotoxicity assessment in other cell types have shared the information in a database for general information.

For ecotoxicological tests certain solvents were used (e.g. DMSO, ethanol, methanol) for the preparation of the stock solutions and then the working solutions were prepared considering the max permitted solvent concentration in the final exposure solutions as indicated in the guideline of each specific assay performed (e.g. 1% or 0.1%, etc).

3. Human Health effects of BPA alternatives

3.1 Methods and results

For Human Health, five endpoints have been prioritized based on the Chemicals Sustainability Strategy and existing information regarding the prioritized BPA alternatives was gathered on the literature performed to identify data gaps (Mhaouty-Kodja et al., 2025):

- Metabolic Fate and Bioactivation Studies

- Endocrine Disruption

- Immunotoxicity

- Developmental Toxicity

- Carcinogenicity

Next, a brief introduction regarding the endpoints and assays performed, followed by the results obtained so far (or in progress/expected) are presented. Further methodological details can be found in Annex A.3.

3.1.1 Metabolic Fate and Bioactivation Studies

The elucidation of metabolic pathways and bioactivation routes is critical for a sound assessment of hazard and risk of substances. Understanding how a substance is metabolized within an organism helps determine its toxicological impact, including the formation of reactive metabolites that could pose a risk to health. Such knowledge is fundamental for making informed decisions regarding safety standards, regulatory measures, and risk management, as recognized by OECD and regulatory agencies. These challenges are being addressed in this task, and most of the ongoing activities are described below.

Ongoing pilot biotransformation studies by [INRAE (FR)] include performing mass balance analyses in HepaRG cells, followed by radio-HPLC profiling, and using radiolabeled molecules (^{3}**H or** ^{14}**C**) to identify key metabolites and quantify the biotransformation of parent compounds (primary and secondary metabolites). To date, all prioritized molecules, except BPS-MAE, have been obtained as radiolabeled standards, with ongoing purification and stability testing. These molecules were either purchased, synthesized or made available by [INRAE (FR)].

To complement these efforts, [ISS (IT)] has developed a diode-HPLC array method (DAD) to detect and quantify the bioavailability of BPA alternatives in *in vitro* assays: BPZ, BPE, BPS-MAE, BPP, BPAP, and TCBPA. The metabolism, using isoform specific enzymes and liver microsomes, and biokinetics studies will provide crucial data for **Physiologically Based Pharmacokinetic (**PBPK) modelling. The data generated - including kinetic parameters, i.e., Km, Vmax, hepatic clearance, - feed directly into quantitative structure-activity relationships (QSARs) and physiologically based exposure models.

The metabolism study has been initiated with BPZ testing various concentrations (5-10-20-40 µM) and incubation times (0-10-20-30-60 min). No significant plastic adsorption on polypropylene safe-lock tubes, used for centrifugation, was observed. However, reliable metabolic data could not be obtained due to BPZ instability in the incubation buffer and low solubility in MeOH - a solvent required for incubation with recombinant enzymes and Human Liver Microsomes (DMSO was avoided as it may interfere with enzymatic activity). The next step will be to evaluate BPE, which shows better (higher) solubility in water matrix, to obtain metabolic kinetic data.

The ongoing work of this endpoint performed by both partners is summarized in Table 27.

Table 27: Summary of the metabolism study from radio-HPLC profiling by [INRAE (FR)] and diode-HPLC array method (DAD) by [ISS (IT)], both in HepaRG cells. Blue, completed. Light blue, ongoing, Yellow planned for PARC Year 4 –until PARC M54 (May 2025 – October 2026)

Substance	**Viability test in HepaRG**	**Radio-labeled - Availability of standards - Purity & stability confirmation over time**	**Radio-labeled Mass balance study in HepaRG cells**	**Radio-labeled Radio-HPLC profiling (quantification of metabolites)**	**Radio-labeled Structural identification of major metabolites**	**Radio-labeled Results summary** Further determine kinetic constants (Km, Vm) for major metabolites	**Isoform specific metabolism study at 5-10-20-40 µM, and incubation times 0-10-20-30-60 min [ISS (IT)]**
BPA	*Completed*	*Ongoing*				May 2026	
BPZ						May 2026	Stability problems in MeOH, DMSO can interfere with enzymatic activity

							No reliable data obtained
BPE						May 2026	*Ongoing*
BPS-MAE						**Radio-labeled molecule availability remains uncertain**	
Pergafast 201						May 2026	
BPP						May 2026	
BPAP						May 2026	
TCBPA						May 2026	
TBBPA						May 2026	

3.1.2 Endocrine Disruption

The endocrine system plays a crucial role in development, regulating growth, metabolism, reproduction, and overall homeostasis in the body. Because it relies on precise communication between organs, any interference can have widespread effects on the entire organism, making endocrine disruption a key concern for hazard and risk assessment. In this context, in vitro studies are being conducted to assess the effects of prioritized BPA alternatives on steroidogenesis, using the OECD TG 456, and thyroid hormone regulation, are being performed.

- Steroidogenesis Assessment by [ISS (IT)]:

The Human H295R Steroidogenesis Assay (OECD TG 456) was used to evaluate the ability of BPA alternatives to alter synthesis of the key steroid hormones—17β-estradiol (E2) and testosterone (T)—in human adrenal cells. Preliminary results from two out of three biological replicates indicate a dose-dependent induction of E2 and inhibition of T levels. Further hormone level assessments are in progress.

Cytotoxicity assessment in human adrenal cells has been completed for all eight prioritized BPA alternatives, using BPA as a reference compound, obtaining the following relative cytotoxicity ranking: **BPP > BPZ > BPS-MAE > TBBPA> BPAP > BPA, BPE, TCBPA.**

All the described data is summarized in Table 28 below.

Table 28: Preliminary results of Human H295R Steroidogenesis Assay (OECD TG 456), conducted by [ISS (IT)]. Blue, completed. Light blue, ongoing,

Substance	Cytotoxicity IC_{50} [µM]	Steroidogenesis assay		
		Max concentration tested [µM]	E2	T
BPA	> 100	50	↑ 33% induction	↓ 90% inhibition
BPZ	37,76 ± 4,57	10	↑ 22% induction	↓ 60% inhibition
BPE	> 100	50	↑ 37% induction	↓ 90% inhibition
BPS-MAE	57,13 ± 4,29		*Ongoing*	
Pergafast 201				
BPP	26,43 ± 3,17			
BPAP	63,37 ± 7,02			

TCBPA	> 100			
TBBPA	61,88 ± 2,42			

- Thyroid Hormone Transport Disruption: T3 by MCT8 and I uptake by NIS [ISCIII (ES)]:

The potential of BPA alternatives to interfere with thyroid hormone transport is being assessed through in vitro assays currently under consideration by EURL ECVAM / NETVAL (validation process ongoing, see Table 29 for preliminary results). These assays evaluate:

- **Cytotoxicity assessment** of **MDCK1 cells** (via morphological alterations): completed for all BPA alternatives (Table 29).
- MCT8 is a highly selective transporter of thyroid hormones, facilitating their entry into various tissues, including the brain, kidney, liver, and heart, thereby regulating neurological health and systemic metabolic balance. **Triiodothyronine (T3) transport via MCT8 transporter** in **MDCK-h- MCT8 cells** (measuring T3 accumulation). **T3 transport inhibition** was tested in at least two replicates for all compounds.
- **Iodide (I-) uptake via the NIS transporter**, a key step in thyroid hormone synthesis. I- transport inhibition was evaluated for two out of 13 substances.

Table 29. Preliminary results of thyroid regulation disruption assays. Blue, completed. Light blue, ongoing.

Substance	Morphological Cytotoxicity (1h incubation) start at [µM]	Max concentration tested [µM]	T3 transport by MCT8 IC_{50} range [µM]	I- transport by NIS IC_{50} range [µM]
BPA	> 250	1000	200-300	200-400
BPZ	> 250	1000	600-700	*Ongoing*
BPE	No	1000	300-400	
BPS-MAE	> 250	1000	No Inhibitor	
Pergafast 201	> 100	1000	800-100	
BPP	> 250	1000	> 1000	
BPAP	> 250	1000	100-200	
TCBPA	No	1000	200-300	
TBBPA	No	1000	800-1000	No Inhibitor
BPH	> 250	1000	No Inhibitor	
BPS	> 250	1000	700-800	
BAGDE	> 250	1000	700-800	
Trans-CBDO	> 250	1000	No Inhibitor	
Positive Control Silychristin (Potent inhibitor)		1000	0,5-2,0	N/A
Positive Control ClO^{4-} (Potent Inhibitor)			N/A	1-5

3.1.3 Immunotoxicity

BPA and its analogues have the potential to disrupt or modulate immune function, potentially increasing susceptibility to infections and altering inflammatory responses. Therefore, understanding how BPA analogues affect immune regulation is relevant for assessing their full impact on human health. Scientifically valid in vitro and in silico methods in line with the OECD Detailed Review Paper No. 360 on In Vitro Test Addressing

Immunotoxicity with a Focus on Immunosuppression were used (https://www.oecd.org/en/publications/detailed-review-paper-on-in-vitro-test-addressing-immunotoxicity-with-a-focus-on-immunosuppression_667965bc-en.html). One of the methods used, based on Jurkat T cells and IL-2 production, is similar to the recently accepted IL-2 Luc Assay (OECD TG444A). (OECD (2023), Test No. 444A: In Vitro Immunotoxicity: IL-2 Luc Assay, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/27b10ba3-en.)

- Immune Cell Targets and Function [UMIL (IT)]:

The effect of BPA analogues on immune function is being investigated using human peripheral blood mononuclear cells (PBMCs) obtained from healthy male and female donors. Initial **cytotoxicity screening** (LDH assay) defined non-toxic concentrations for further analysis (Tables 30 and 31). The study focuses on:

- **Antibody production and Natural Killer cell (NK-cell) activity:** BPA and its analogues were tested for their ability to alter B cell differentiation and antibody production (crucial for identifying, neutralizing, and marking pathogens for destruction) as well as natural killer (NK) cell lytic activity, involved in innate immunity defense the first-line response to infections and tumors. While the results show at non-cytotoxic concentrations (cell viability > 80%) a reduction in the release of both IgG and IgM for all tested substances, with differences based on sex and the specific immunoglobulin examined, no changes in NK-cell lytic activity were detected.
- **T-cell proliferation and differentiation:** Ongoing analyses are examining BPA alternatives' on T-cell activation markers and cytokine production, which are central to the activation of adaptive immune responses and critical for infection resistance and immune regulation.

Table 30: Summary of data from *in vitro* assay in PBMCs of healthy donors by UMIL (IT). Blue, completed. Light blue, ongoing.

Substance	NK-cell lytic activity PBMC 24h exposure + co-culture with K562 for 4h	T-cell proliferation and differentiation PBMC 24h exposure + 4 days with anti-CD3 and anti CD28	
		Male donors	Female donors
BPA	No significant changes compared to controls	*ongoing*	*ongoing*
BPZ		*ongoing*	*ongoing*
BPE		*ongoing*	*ongoing*
BPS-MAE		*ongoing*	*ongoing*
BPP		*ongoing*	*ongoing*
BPAP		*ongoing*	*ongoing*
TCBPA		*ongoing*	*ongoing*

Table 31: Summary of data from in-vitro assays in PBMCs of healthy donors by UMIL (IT). Any value shown in the graph represents the mean ± SD, with n = 5 males and n = 5 females. Only statistically significant results are reported.

Substance	Determination of cell viability of 80% (CV80) PBMC 24h exposure CV80 [µM]		T-cell independent antibody production PBMC 24h exposure + 6 days of stimulation with ODN2006 and IL-2		
			Effect on IgG and IgM release		IC_{50} [µM]
	Male donors	Female donors	IgG	IgM	No genders difference

BPA	50,84	54,37	↓ 50 µM female	↓ 50 µM male	38,29 ± 0,17
BPZ	13,81	17,82	↓ 15 µM female 1,5 µM male vs female	↓ 0,15 and 15 µM male	14,50 ± 0,12
BPE	51,87	59,33	↓ 50 µM female	↓ 50 µM female	41,74 ± 0,40
BPS-MAE	35,80	60,65	↓ 35 µM male ↓ 60 µM female	↓ 0,35, 3,5 and 35 µM male ↓ 60 µM female	33,22 ± 0,71
BPP	13,13	20,44	↓ 15 µM female 0,15 and 1,5 µM male vs female	↓ 1,5 and 15 µM male ↓ 15 µM female 15 µM male vs female	9,89 ± 2,06
BPAP	12,90	15,74	↓ 15 µM female 0,15 µM male vs female	↓ 15 µM male and female 15 µM male vs female	12,42 ± 0,77
TCBPA	17,59	30,23	↓ 25 µM female 2,5 and 25 µM male vs female	↓ 25 µM male	23,41 ± 2,25

- Immunometabolism and Inflammation Pathways [MUI (AT)]:

- **Tryptophan metabolism:** Given critical role of tryptophan in immune regulation, BPA analogues' effects on inflammation-induced tryptophan breakdown are being investigated in human PBMCs and monocyte-derived cell lines by measuring the concentrations of tryptophan and its catabolite kynurenine. Experiments are still ongoing.

- **Metabolic markers:** Proliferation assays and gene expression profiling provide insight into immunometabolic disruptions induced by BPA alternatives. Metabolic activity/viability was assessed by using resazurin as a probe. Primers were designed for relevant metabolic enzymes. Experiments for primer validation and cell treatments for RNA collection have been initiated.

- Immunomodulation and Targeting the Glucocorticoid Receptor [UL FFA]:

The effect of selected BPA analogues on the metabolic activity and the release of cytokines from in vitro models of human macrophages, T cells and B cells is being evaluated. Preliminary results point to **BPP, BPAP, and BPZ as the most cytotoxic, with BPP showing IC50 values five times lower than BPA** (Table 31).

In silico screening (Endocrine Disruptome): a computational analysis helped to identify potential interactions of BPA alternatives with 12 hormone nuclear receptors, including the glucocorticoid system, by ranking BPA analogues based on their likelihood to bind to the receptors. **BPS-MAE, PF201, BPZ, BPAP, and BPP exhibited higher binding potential (to all 12 nuclear receptors) than BPA, while BPE and TCBPA were less active**. These results are gathered in a manuscript currently under revision.

Glucocorticoid receptor (GR) signalling: to determine immunomodulatory potential; relevant for stress & hormone modulation. Experimental evaluations using macrophages (THP-1 derived), T cells (Jurkat cells), and B cells (lymphoblastoid cell lines, LCLs) assess how BPA analogues influence cytokine release and GR activation:

- **Macrophages (THP-1 derived):** IL-1β was the most affected cytokine, showing **biphasic modulation by BPP** (stimulation at nanomolar doses, suppression at micromolar doses). IL-6 release was increased by **BPS-MAE, BPZ, TCBPA, and PF201**, while TNF-α remained unchanged and only 10 micromolar TCBPA stimulates the release of IL-8.

- **T-cell responses (Jurkat cells): BPA analogues generally suppressed IL-2 release in low micromolar range** (a classic marker for T cell activation), except for TCBPA, which stimulated IL-2 at low micromolar concentrations but suppressed it at higher doses.
- **B cells (Lymphoblastoid, LCLs):** IL-2, IL-6, and IL-10 were suppressed by most BPA analogues at 10 µM, while TCBPA again stood out with different behaviour by IL-6 is increased by 10 uM TCBPA went rest of cytokines are mostly downregulated. Comparably to the results in THP-1 derived macrophages, TNFα was the least affected cytokine.

The data described is summarized in a manuscript, which has been submitted to Ecotoxicology and Environmental Safety journal and is currently under review.

Next steps

- qRT-PCR protocol for glucocorticoid receptor modulation in THP-1 derived macrophages is being established.

- Developing a stable GR-expressing T cell line (Jurkat cells) that will enhance mechanistic studies.

Table 32: Summary of data from in-vitro cytotoxicity assays in macrophages (THP-1 derived), T cells (Jurkat cells) and LCLs viaresazurin method for24h exposure, n=3 by UL FFA. Blue, completed. Light blue, ongoing.

Substance	Jurkat T cells Cytotoxicity IC50 [µM]	THP-1 derived macrophages Cytotoxicity IC50 [µM]	Lymphoblastoid cell lines (LCLs) Cytotoxicity IC50 [µM]
BPA	99,4 ± 5	122,5 ± 3,2	182,8 ± 16,1
BPZ	38,1 ± 1	44,8 ± 2,1	65,9 ± 4,9
BPE	208,2 ± 6	208,5 ± 4,6	220,2 ± 11,2
BPS-MAE	141,4 ± 7	145,2 ± 8,0	226,8 ± 34,5
Pergafast 201	148,1 ± 7,5	98,2 ± 5,6	178,8 ± 0,7
BPP	11,1 ± 0,1	18,8 ± 0,5	28,6 ± 1,5
BPAP	26,5 ± 0,9	36,9 ± 0,7	62,4 ± 8,5
TCBPA	41,5 ± 3	73,1 ± 3,4	89,8 ± 4,6
TBBPA			

- Calcium Homeostasis and Mechanistic Insights [ISS (IT)]:

Calcium signalling is critical to immune cell activation. To investigate the pathways involved in immunotoxicity, ongoing studies are evaluating BPA analogues' effects on intracellular calcium homeostasis in T cells (Jurkat cells). Short and long-term effects of the selected BPA analogues on both resting intracellular calcium concentration ($[Ca^{2+}]_i$) and regulated calcium responses are being evaluated. The findings will help highlight the potential of these substances to trigger and/or disrupt key calcium-mediated cellular events, which are central to T-cell activation. Preliminary results indicate (Table 33):

BPP, BPAP, and BPZ significantly increase basal $[Ca^{2+}]_i$, while inhibiting the responses following T-cell receptor (TCR) activation.

Effects are dose-dependent, occurring at lower concentrations than those affecting cell viability.

Serum proteins (e.g., albumin) reduce BPZ potency, suggesting that the effect is linked to the analog's free fraction**, and that binding interactions might affect toxicity.**

Table 33: Summary of preliminary results from intracellular calcium homeostasis assays in Jurkat T cell line [ISS (IT)] Blue, completed. Light blue, ongoing. Blank row Not performed and not planned.

Substance	Acute effects (Whitin 5 min exposure)			Long-term effects (24h)		
	Max concentration tested (µM)	Basal $[Ca^{2+}]_i$	$[Ca^{2+}]_i$ response to TCR activation	Max concentration tested (µM)	Basal $[Ca^{2+}]_i$	$[Ca^{2+}]_i$ response to TCR activation
BPA	100	*ongoing*	*ongoing*	-	*planned*	*planned*
BPZ	100	↑	↓	20	*ongoing*	*ongoing*
BPE	100	*ongoing*	*ongoing*	-	*planned*	*planned*
BPS-MAE	-	*planned*	*planned*	-	*planned*	*planned*
Pergafast 201						
BPP	10	↑	↓	-	*planned*	*planned*
BPAP	100	↑	↓	-	*planned*	*planned*
TCBPA	50	*ongoing*	*ongoing*	-	*planned*	*planned*
TBBPA						

Conclusions on immunotoxicity

Table 34 presents the key findings from tests assessing the immunotoxicity of BPA alternatives on human health.

Table 34: Summary of Immunotoxicity Findings of BPA Alternatives

Immune Function	Key Finding	Interpretation	Regulatory Relevance
T-Independent Antibody Production (B Cells – PBMCs, LCLs)	BPA analogues **reduced IgM and IgG production**, with dose- and **sex-dependent effects** (IgG suppression in females, IgM suppression in males).	**B-cell dysregulation** may impair **adaptive immunity** weaking immune protection.	Reduced antibody production could affect **vaccination response** and **infection resistance**, highlighting a **risk of immunosuppression**.
NK-Cell Cytotoxicity (PBMCs – NK Cells)	**No significant alterations** in NK-cell lytic activity across tested substances.	NK-cell function appears **resilient** to BPA analogue exposure, meaning innate immune surveillance is **not directly impaired**.	While no immediate concern, **long-term effects** and interactions with other immune stressors require further study.
T-Cell Proliferation & Differentiation (PBMCs – CD4+/CD8+ T Cells, Jurkat Cells)	BPA alternatives **suppressed IL-2 release**, except for **TCBPA**, which showed **biphasic effects** (stimulated at low doses, suppressed at high doses).	**IL-2 suppression suggests impaired T cell activation**, which could lead to **immune suppression**. The **biphasic response of TCBPA** suggests that **dose-dependent effects** influence immune function differently.	IL-2 suppression raises concerns about **T cell anergy**, potentially affecting **pathogen defense and autoimmune balance**. Further evaluation is needed for **low-dose effects**.
Glucocorticoid Receptor (GR) Signaling & Cytokine Modulation	- **BPP, BPAP, and BPZ were the most cytotoxic**. - **IL-1β showed biphasic modulation** by BPP (stimulation at	**GR signaling disruptions may lead to immune suppression or excessive inflammation**. **BPP's biphasic effect on IL-1β suggests a shift**	**GR disruption is a major regulatory concern** due to its role in **stress response and immune homeostasis**. IL-6 elevation suggests potential **chronic inflammatory risks**,

(Macrophages, T Cells, LCLs)	nanomolar doses, suppression at micromolar doses). - **IL-6 increased** with BPS-MAE, BPZ, TCBPA, and PF201. - **TNF-α was largely unchanged**.	**between inflammation activation and suppression**. IL-6 elevation indicates a **risk of chronic inflammation or autoimmunity**.	supporting the need for **further evaluation of exposure limits**.
Calcium Homeostasis in Immune Cells (T Cells – Jurkat)	- **BPP, BPAP, and BPZ increased basal calcium levels** but **inhibited T-cell receptor (TCR) activation responses**. - The **presence of albumin reduced BPZ-induced calcium effects**, suggesting protein binding affects toxicity.	**Disruptions in calcium signaling can impair T cell activation**, leading to **weakened immune responses** or **dysregulated cell signaling**.	**Mechanistic evidence of immunotoxicity** – calcium homeostasis disruption is a **recognized marker of immune dysfunction**. These findings support **AOP development** for immune dysregulation.

3.1.4 Developmental Neurotoxicity

The developing nervous system is particularly sensitive to chemical exposures, making developmental neurotoxicity (DNT) a significant concern for human health. To evaluate the potential neurotoxic effects of BPA alternatives, two complementary *in vitro* strategies are being applied, aiming to improve mechanistic understanding of how BPA alternatives disrupt neurodevelopmental processes These include hippocampal differentiation and neuronal proliferation, differentiation, and migration, neurite outgrowth, and mitochondrial function.

- Hippocampal neurons in vitro screening by [CNRS (FR)]:

This is an early-stage assay, under development to complement the established DNT *in vitro* battery (IVB), which includes assays for all the processes listed above. The DNT-IVB has gone through an initial evaluation by the OECD (OCDE, 2023). The DNT-IVB lacks endpoints for hippocampal development and function.

By developing a method for differentiating hippocampal neurons from hiPSCs as a new complementary approach within the DNT-IVB framework, this new test system will enable targeted assessment of substances affecting hippocampal development and function. The hippocampus is a key brain region involved in learning and memory and a critical driver of cognitive abilities.

Differentiation Process: Two hiPSC clones were successfully differentiated into hippocampal neural progenitors (hNPCs) and matured into neurons over 43 days. Key markers confirmed successful differentiation:

- Neuronal Progenitor Cells marker: Nestin
- Hippocampal progenitor markers: PROX1, ZBT20
- Neuronal markers: MAP2, ELAVL2, GRIK4

Preliminary *in vitro* screening revealed, that exposure of hNPCs to **BPA, BPAP, BPE, BPZ** at seven concentrations ranging from 0,001 to 100 µM (14-day exposure) resulted in **concentration-dependent decrease in cell viability**, The decrease was statistically significant at 100 µM **for BPA, BPAP, BPZ**, and from **10 µM for BPE**.

Subsequent experiments will study:

- Cellular morphology: β-III tubulin immunocytochemistry (neurites per neuron, branch length), neuronal network formation.
- Synaptic Plasticity markers: Western blot for synapsin 1, PSD95, gephyrin**,** synaptogenesis

- Gene expression and hormonal pathways: receptors for sex steroids and thyroid hormones (TH) and DNA methylation extracted from molecular analysis

Table 35. Test system enabling targeted assessment of substances affecting hippocampal development and function

Substance	Cell viability	at 0.001, 0.01, 0.1, 1 µM, 14-day incubation						
		Neuronal morphology β-III tubulin			Pre and Post synaptic markers			Gene expresion analysis
		neurites x neuron	Length	Branches x neuron	synapsin1	PSD95	gephyrin	
BPA	100 µM	*ongoing*	*ongoing*	*ongoing*	planned	planned	planned	planned
BPZ	100 µM	*ongoing*	*Ongoing*	*ongoing*	"	"	"	"
BPE	10 µM	"	"	"	"	"	"	"
BPS-MAE	planned							
Pergafast 201								
BPP	planned							
BPAP	100 µM	"	"	"	"	"	"	"
TCBPA	planned							
TBBPA								

- Neurosphere Assay by [IUF (DE)]

This is an established high-content, high-throughput model, assessing seven key neurodevelopmental events as part of the DNT-IVB approach. This assay [Koch et al. 2022] is part of the DNT in vitro test battery (DNT-IVB) (DNT-IVB; Blum et al. 2023; OECD377 2023) and evaluates seven key neurodevelopmental events using human fetal neural progenitor cells (hNPCs).

Endpoints Assessed: Proliferation, differentiation, migration, neurite outgrowth, and mitochondrial function by using the following assays:

- Neural progenitor proliferation (NPC1).
- Radial glia, neuronal, oligodendrocyte migration (NPC2a/b/c).
- Neuronal differentiation (NPC3), neurite outgrowth (NPC4), oligodendrocyte differentiation (NPC5).

Experimental design and preliminary findings are summarized below, Table 36:

NPC1 Assay (72h): Neurospheres exposed to 7 concentrations (20–0,027 µM).

NPC2-5 Assays (72h): Neurospheres exposed to 7 concentrations (20–0,027 µM).

plated on ECM for 5-day exposure.

Radial glia migration (NPC2a): Inhibited by BPZ, BPAP, BPS-MAE at low micromolar concentrations.

Neuronal differentiation (NPC3): Impaired at submicromolar doses for BPP and BPAP.

Oligodendrocyte differentiation (NPC5): Strong disruption by BPAP (BMC30: 0.155 µM).

The final classification as specific, unspecific, borderline hit is pending and will be performed after final classification (few evaluations pending). This will be added either in the final report of the BPA project (M54, October 2026) or in the 2nd Data Gaps report (M60, May 2027).

Table 36. Summary of benchmark concentrations (BMCs) across all endpoints of the Neurosphere assay

Substance	BPA	BPAP	BPE	BPP	BPZ	BPS-MAE	TCBPA	TBBPA*
Cytotoxicity (NPC1)	No Hit	No Hit	No Hit	No Hit	No Hit	No Hit	No Hit	No Hit
Mitochondrial activity (NPC1)	No Hit	No Hit	No Hit	No Hit	No Hit	No Hit	No Hit	No Hit
NPC proliferation 72h (NPC1)	No Hit	No Hit	No Hit	No Hit	No Hit	No Hit	No Hit	No Hit
Cytotoxicity 72h (NPC2-5)	No Hit	BMC_{10}: **7.30µM**	No Hit	BMC_{10}: **3.03µM**	BMC_{10}: **8.65µM**	No Hit	BMC_{10}: **2.43µM**	BMC_{20}: **1.75µM**
Radial glia migration 72h (NPC2a)	No Hit	BMC_{10}: **1.72µM**	No Hit	BMC_{10}: 3.77µM	BMC_{10}: **0.896µM**	BMC_{10}: **<0.027µM**	BMC_{10}: **1.16µM**	BMC_{20}: **1.75µM**
Cytotoxicity 120h (NPC2-5)	No Hit	BMC_{10}: **6.11µM**	No Hit	BMC_{10}: **2.40µM**	BMC_{10}: **6.82µM**	No Hit	BMC_{10}: **2.80µM**	BMC_{20}: **0.63µM**
Mitochondrial activity 120h (NPC2-5)	No Hit	BMC_{30}: **4.97µM**	No Hit	BMC_{30}: **3.14µM**	BMC_{30}: **7.97µM**	No Hit	BMC_{30}: **1.94µM**	BMC_{20}: **1.38µM**
Radial glia migration 120h (NPC2a)	No Hit	BMC_{10}: **3.81µM**	No Hit	BMC_{10}: **1.87µM**	BMC_{10}: **3.99µM**	No Hit	BMC_{10}: **2.89µM**	BMC_{20}: **1.93µM**
Neuronal migration 120h (NPC2b)	No Hit	BMC_{30}: **8.22µM**	No Hit	BMC_{30}: **>2.22µM**	BMC_{30}: **6.69µM**	No Hit	BMC_{30}: **6.16µM**	BMC_{20}: **2.6µM**
Oligodendrocyte migration 120h (NPC2bc)	No Hit	BMC_{30}: **8.56µM**	No Hit	BMC_{30}: **>2.22µM**	BMC_{30}: **15.8µM**	No Hit	BMC_{30}: **4.10µM**	BMC_{20}: **2.23µM**
Neuronal differentiation 120h (NPC3)	No Hit	No Hit	No Hit	BMC_{30}: **0.691µM**	BMC_{30}: **>6.66µM**	No Hit	BMC_{30}: **6.64µM**	BMC_{20}: **2.18µM**
Subneurite length per nucleus 120h (NPC4b)	No Hit	No hit (N=1)	No Hit (N=2)	BMC_{30}: **>2.22µM**	BMC_{30}: **>6.66µM**	No Hit (N=2)	BMC_{30}: **>6.66µM** (N=1)	BMC_{20}: **2.31µM**
Mean neurite area without nuclei 120h (NPC4a)	No Hit	BMC_{30}: **8.70µM** (N=1)	No Hit (N=2)	BMC_{30}: **2.27µM**	No Hit	No Hit (N=2)	BMC_{30}: **>6.66µM** (N=1)	BMC_{20}: **2.49µM**
Oligodendrocyte differentiation 120h (NPC5)	No Hit	BMC_{30}: **0.155µM**	No Hit	BMC_{30}: **>2.22µM**	BMC_{30}: **>6.66µM**	No Hit	BMC_{30}: **>6.66µM**	BMC_{20}: **0.55µM**

The endpoint analysis was performed as a percentage of the solvent control, and different benchmark response values were used as described in Blum et al 2022 and indicated as subscript character. BMCs were calculated using the CRStats software (available at https://github.com/ArifDoenmez/CRStats), e.g., BMC_{10} is defined as the concentration, that reduces or increases the respective response to 90% or 110% of the solvent control. No hit indicates that the respective BMR was not reached. * TBBPA results were obtained in Klose et al 2021 using different BMRs. Classification of results in specific, unspecific and borderline hits is still pending.

3.1.5 Carcinogenicity

Carcinogenicity refers to a chemical's potential to cause cancer through genotoxic or non-genotoxic mechanisms. Given its critical implication for public health, regulatory agencies emphasize rigorous assessment of this risk (EFSA Scientific Committee; Scientific Opinion on genotoxicity testing strategies applicable to food and feed safety assessment. 2011). To ensure safety of prioritized BPA alternatives, in vitro assays are being conducted to evaluate both genotoxic and non-genotoxic carcinogenicity.

The three experimental approaches planned to assess genotoxic and genotoxic carcinogenicity were developed as follows:

- **Genotoxicity Carcinogenicity Testing:**

Approach I. A battery of in vitro genotoxicity tests, aligned with EFSA recommendations, is being conducted using harmonised Standard Operation Procedures (SOPs) – bacterial gene mutation test (Ames test) and in vitro micronucleus assay in mammalian cells

- Bacterial Gene Mutation Assay (Ames test, OECD TG471 by [ISS (IT)]) was conducted in *Salmonella* Typhimurium strains TA98, TA100, and TA102. Five concentrations of BPE and BPP, ranging from 100 to 5,000 µg/plate, were tested in two independent experiments, both with and without metabolic activation. **No significant toxicity or increase in revertant colonies** was observed, indicating **no mutagenic potential** under the test conditions.

These are preliminary data. TA98 and TA100 detect the same type of DNA damage identified by TA1537 and TA1535 but are supposed to be more sensitive. Therefore, we started the analyses with this set of strains. We will complete the study with all the five requested strains for REACH / regulatory purposes.

- Micronucleus Assay in Mammalian Cells (OECD TG487) by [ISS (IT), INSA (PT)].

To assess the potential for chromosomal damage, the in vitro micronucleus test was conducted in human peripheral blood lymphocytes. Preliminary results have been obtained for **BPE, BPP, BPAP and BPS-MAE**, with and without metabolic activation. **Findings are presented in Table 37 and highlights are indicated below:**

BPE (1.25–100 µM): Mild cytotoxicity at **100 µM**; **significant micronuclei induction** after continuous treatment **without metabolic activation**.

BPP (1.25–100 µM): No severe cytotoxicity, but an **increased frequency of micronuclei** after short treatment **without metabolic activation**.

BPAP (1–640 µM): Cytotoxic at high concentrations; no genotoxic effects detected.

BPS-MAE (1–640 µM): Cytotoxic at high concentrations; **no significant micronuclei induction** observed.

Table 37. Summary of data from Micronucleus assay following OECD TG487 in human lymphocytes. Yellow, ongoing assays. n refers to the number of independent experiments. Partners will perform a 2nd experiment for each substance.

Substance	24h without S9	3h with S9	3h without S9	Results summary	Partner
BPA					ISS / INSA
BPZ				Nov 2023-Dec 2025 **Done:** dose-finding experiments / cytotoxicity tests (MTT, CBPI)	INSA
BPE	n=2	n=2	n=2	Oct 2023-Dec 2025 **Done:** cytotoxicity tests (trypan blue, CBPI), 8 concentrations, (range 1,25-100 µM) in the presence and absence of metabolic activation	ISS
BPS-MAE	n=1	n=1	n=1	Nov 2023-Dec 2025 **Done:** dose-finding experiments / cytotoxicity tests (MTT, CBPI)	INSA
Pergafast 201					
BPP	n=1	n=1	n=1	Oct 2023-Dec 2025 **Done:** cytotoxicity tests (trypan blue, CBPI), 8 concentrations, (range 1,25-100 µM) in the presence and absence of metabolic activation	ISS
BPAP	n=1	n=1	n=1	Nov 2023-Dec 2025 **Done:** dose-finding experiments / cytotoxicity tests (MTT, CBPI)	INSA
TCBPA				Nov 2023-Dec 2025 **Done:** dose-finding experiments / cytotoxicity tests (MTT, CBPI)	INSA

TBBPA					

Approach II. **Advanced Hepatic Cell Models. Micronucleus, comet and transcriptomic assays in 2D and 3D HepG2 cells by [Fraunhofer (DE), NILU (NO), INSA (PT)]:**

To enhance physiological relevance to humans, 2-D and 3-D HepG2 cell models are being used to evaluate chromosomal integrity (Micronucleus assay), DNA strand breaks (Comet assay) and gene expression profiling (transcriptomic analysis).

Dose-range findings has been completed for six prioritized BPA alternatives and adaption of the micronucleus assay in 3-D HepG2 cells is ongoing.

Non-genotoxic carcinogenicity testing

Approach III. Bhas 42 cell transformation assay (CTA):

To assess non-genotoxic carcinogenicity, the Bhas 42 Cell Transformation Assay (CTA) (OECD TG 231 by [NILU (NO), INSA (PT), TTL (AT), INRS (FR)]) is being applied in alignment with OECD guidance document to investigate tumor initiation and promotion. SOPs have been prepared (see Annex A.3), and inter-laboratory comparisons have been conducted to harmonize protocols across partners. Preliminary dose-range finding and CTA results have been obtained for three BPA alternatives, with testing ongoing for the remaining substances. The first results from the protocol harmonization, using the selected controls, were consistent across all participating labs. Each substance is being tested by two different laboratories, except BPA, which has been tested by the four laboratories involved (Table 38).

Table 38 - Summary of data from CTA in Bhas cells. n refers to the number of independent experiments. Partners will perform a 2nd experiment for each substance.

Substance	Initiation					Promotion				
	Max concentration tested (µM)*	Partner 1	Partner 2	Partner 3	Partner 4	Max concentration tested (µM) *	Partner 1	Partner 2	Partner 3	Partner 4
BPA	100	**n = 1**	**n = 2**	**n = 1**	**n = 1**	100	**n = 1**	**n = 2**	**n = 1**	**n = 1**
BPZ	25			**n = 1**		25			**n = 1**	
BPE	50		**n = 2**	**n = 1**		50		**n = 2**	**n = 1**	
BPS-MAE**	50				**n = 1**	100				**n = 1**
BPP	10		**n = 1**		**n = 1**	10		**n = 1**		**n = 1**

BPAP	25 - 40	**n = 1**			**n =1**	25 - 32.5	**n = 1**			**n =1**
TCBPA	50	*planned*		**n = 1**		50	*planned*		**n = 1**	

*cell death observed at higher concentrations; ** 2nd partner to be definedEnvironmental effects of BPA alternatives

In contrast with the Human Health studies, the Environmental work consist mostly of whole organism assays that covers the adverse effects (e.g. apical endpoints such as mortality, effects related to the anticipated mode of action or MIE, effects on reproduction or effects on the endocrine system) of BPA alternatives on whole organisms belonging to different taxa.

The main objective of this part of the project (P5.1.2.b_Y1_BPAalternatives_BPI_MU) was to

- fill in knowledge gaps in the toxicity of specific bisphenols and

- investigate the possible adverse effects on organisms belonging in different taxa when they will be exposed under realistic conditions in environmental relevant concentrations of mixtures of bisphenols as a retrospective assessment.

This deliverable is focusing on the investigation of the toxicity of single bisphenols although the experiments with mixtures have been started by certain partners. The testing strategy has been discussed and agreed with all involved partners considering each partner's capacity and expertise and summarised in tables in Annex A.4.

It is very important to highlight that all selected bisphenols from group 2 and group 3, Table 25, will not be tested for all organisms and tests. In certain cases, the toxicity assessment is under industry's responsibility since bisphenols are regulated under REACH and the information requirements are set according to the tonnage level in which the substance is manufactured or imported per year (*e.g.* 1-10 t/y, 10 – 100 t/y, >100 t/y, >1000 t/y, etc) (Annexes VII to X).

As regards the testing strategy for mixtures, it is presented in landmark L- BPAalternatives_ENV.3 and partner UFZ(DE) is responsible for the design of the mixtures to be tested.

4.1 Testing methods

The testing systems are summarized per organism in the Table 39 and detailed below (Tables in Annex A.4):

- Aquatic invertebrates

Partners contributing: BPI (EL), IISPV (ES), IEP-NRI (PL), UG-PL (PL), UAVR (PT), SU (SE), INERIS (FR), SDU (DK), SLU (SE), Cefas (UK), UFZ(DE)

The tests for the toxicity assessment of aquatic invertebrates were carried out according to the following testing guidelines:

- Acute toxicity test on *Daphnia magna* (OECD TG 202)
- *Daphnia magna* reproduction test (OECD TG 211)
- Short-term Juvenile Hormone Activity Screening Assay (JHASA, OECD proposal)
- *Lymnaea stagnalis* Reproduction Test (OECD TG 243)
- *42-day chronic toxicity test using the freshwater amphipod Hyalella azteca, spiking sediment with BPAF*

- *10-day acute sediment toxicity bioassay with the marine polychaete Arenicola marina.* In this assay, surviving organisms were used for the following biomarkers analysis: Cellular Energy Allocation (CEA) and Glutathione S-transferase (GST)
- Regeneration assay - freshwater planarian *Girardia tigrine*

- Zebrafish

Partners contributing: INERIS (FR), UAVR (PT), SDU (DK), BPI (EL), NIB (SI)

The tests for the toxicity assessment of zebrafish were carried out according to the following testing guidelines:

- Fish Embryo Acute Toxicity Test (OECD TG 236)

In certain cases in the above mentioned protocol, additional endpoints were investigated: THS-sensitive endpoints such as inflation of the posterior swimbladder, eye development including the area of the retinal pigment layer (RPE), and swimming performance analysis.

- EASZY assay: Detection of substances acting through Estrogen Receptors Using Transgenic cyp19a1b GFP Zebrafish Embryos (OECD TG 250).

As regards INERIS (FR), it was initially planned to assess the endocrine and reproductive effect of one bisphenol substitute in the OECD TG 229 but it was not possible to perform this experiment due to unforseen reasons. In place of performing the TG 229, the partner assessed the metabolic endocrine activity of some bisphenol substitutes in a novel zebrafish embryo assay, called *EMERGE* (Effect of metabolic endocrine disruptors in the Gut of zebrafish embryos) which allows to evaluate the effect of chemicals on the intestinal expression of the cyp3a65 gene. The EMERGE assay is not yet endorsed by OECD and was developed within the Horizon EU project OBERON.

Partner UU (SE) initially planned to perform tests on zebrafish to assess the metabolic disruption in fish but due to unforeseen reasons it was not possible at least for Y1 & Y2.

- Alga / Aquatic plants

Partners contributing: IISPV (ES), IEP-NRI (PL), BPI (EL), UG-PL (PL), SU (SE)

The tests for the toxicity assessment of alga and aquatic plants were carried out according to the following testing guidelines:

- Algae growth inhibition test (OECD TG 201)
- *Lemna sp*. Growth Inhibition Test (OECD TG 221)

- Soil organisms

Partners contributing: BPI (EL), IISPV (ES)

The tests for the toxicity assessment of soil organisms were carried out according to the following testing guidelines:

- Earthworm reproduction test (OECD 222)
- Effects on soil micoorganisms: Carbon transformation test (OECD TG 217 and ISO 11274)
- Effects on soil microorganisms: Nitrogen transformation test (OECD TG 216)

- Amphibians

Partners contributing: UAVR (PT)

The tests for the *in vitro* and *in vivo* toxicity assessment of amphibians were carried out according to the following testing guidelines:

- Cytotoxicity on two Xenopus-derived cellular models (A6, XTC-2)
- Frog embryo teratogenicity assay (ASTM E1439-12)

- In vitro / high throughput assays

Partners contributing: BPI (EL), IISPV (ES), IEP-NRI (PL), UAVR (PT), UFZ (DE), EAWAG (CH)

- In vitro YES assay
- Microtox® Acute Toxicity Test
- Fish Cell Line Acute Toxicity - The RTgill-W1 cell line assay (OECD TG249)

Table 39. Comprehensive testing matrix. BPA alternatives and Environmental Assays performed.

		Group 1								Group 2		Group 3			
	Tests	**BPA**	**BPZ**	**BPE**	**BPS-MAE**	**Pergafast 201**	**BPP**	**BPAP**	**TCBPA**	**BPPH**	**BPS-MPE**	**BPB**	**BPF**	**BPS**	**BPAF**
Aquatic invertebrates	Acute toxicity test on *Daphnia magna* (OECD TG 202)	X-X	X	X	P	-	-	-	-	-	-	X – X-X	X - X - X	X -X- X	X
	Daphnia magna reproduction test (OECD TG 211, level 4 of OECD CF)	X	-	-	-	-	-	-	-	-	-	-	X	X	X
	Short-term Juvenile Hormone Activity Screening Assay using *Daphnia magna* (JHASA, OECD 253)	X	-	-	-	-	-	-	-	-	-	X	X	X	-
	Lymnaea stagnalis Reproduction Test (OECD TG 243)	X	X	X-X	X	-	-	X	-	-	-	-	-	-	-
	Regeneration assay using the freshwater planarian *Girardia tigrina*	X	X	-	X	-	-	-	-	-	-	-	-	-	X
	10-day acute sediment toxicity bioassay with the marine polychaete Arenicola marina	X	X	-	-	-	-	X	-	-	-	-	-	-	X
	42-day chronic toxicity test using the freshwater amphipod Hyalella azteca, spiking sediment	-	-	-	-	-	-	-	-	-	-	-	-	-	X
Zebrafish	Fish Embryo Acute Toxicity Test (OECD TG 236)	X-X	X-X	X-X	X-X	-	-	X-X	X	X	X	X-X	X-X	X-X	X-X
	EASZY assay: Detection of substances acting through Estrogen Receptors Using Transgenic cyp19a1b GFP Zebrafish Embryos (OECD TG 250, level 3 of OECD CF)	X	X	X	X	-	-	X	X	-	X	X	X	X	X
Alga / aquatic	Algae growth inhibition test on *Raphidocelis subcapitata* (formerly	X	-	-	-	-	-	-	-	-	-	X	X-X	X-X	X

		Group 1								Group 2		Group 3			
	Tests	**BPA**	**BPZ**	**BPE**	**BPS-MAE**	**Pergafast 201**	**BPP**	**BPAP**	**TCBPA**	**BPPH**	**BPS-MPE**	**BPB**	**BPF**	**BPS**	**BPAF**
	known as *Selenastrum capricornutum*) (OECD TG 201)														
	Lemna sp. Growth Inhibition Test (OECD TG 221)	P	X	X	-	-	X	-	-	-	-	-	P	p	-
Soil organisms	Effects on soil micoorganisms: Carbon transformation test (OECD TG 217 and ISO 11274)	-	O	O	O	O	O	O	-	O	-	-	-	O	-
	Effects on soil microorganisms: Nitrogen transformation test (OECD TG 216)	-	P	P	P	P	P	P	-	P	-	-	-	P	-
	Earthworm reproduction test (OECD 222)	-	O	O	-	-	-	-	-	-	-	-	-	-	-
Amphibians	*In vitro* assays with A6 epithelial-like cells (ECACC 89072613) of *Xenopus laevis*	X	X	X	X	-	-	-	-	-	-	-	-	-	X
	Behaviour toxicity assay with larvae of *Xenopus laevis*	X	X		X	-	-	-	-	-	-	-	-	-	X
	Frog embryo teratogenicity assay (ASTM E1439-12)	X	X	X	X	-	-	-	-	-	-	-	-	-	X
***In vitro* / high throughput assays**	Fish Cell Line Acute Toxicity - The RTgill-W1 cell line assay (OECD TG249)	X	X	X	X	-	X	X	X		X	X	X	X	X
	Microtox® Acute Toxicity Test	-	X	X	X-X	X	X	X	-	X	X-X	-	X-X	-	X-X
	YES assay, *in vitro* screen with *Saccharomyces cerevisiae*	X	X	X	-	-	-	X	-	-	-	-	-	X	X

"x" indicates that the assay was performed for the corresponding substance, in few cases, more than one partner performed the same assay with the same substance.

O: on going

P: planned

4.2 Results per organism

- Aquatic invertebrates

Three partners performed acute (immobilization) toxicity test on *Daphnia magna* (OECD TG 202) [BPI (EL), INERIS (FR)] and SU (SE)] and the results are presented below. There are four other partners [IISPV (ES), IEP-NRI (PL), UG-PL (PL) and UAVR (PL)] with ongoing experiments and/or experiments that will be carried out with mixtures and presented in the next deliverable.

The results by **BPI (EL)** are presented in Table 40 as nominal and measured concentrations:

Table 40. *Daphnia magna* acute immobilization test: The EC_{50} values (48h) both nominal and measured for each bisphenol alternative tested. Nominal and measured values are considered the same when they present a ±20% difference.

Bisphenols from Group 3 tested	EC_{50} range 48hrs/mg/L	
	Nominal	Measured
BPB		
BPF		
BPS		
BPAF		

Red colour: values 1-10 mg/L,
Green colour: values >100 mg/L

Partner **SU (SE)** performed acute and reproduction toxicity assessment on *Daphnia magna* (OECD TG 202 and OECD TG 211 respectively). The results are presented in Table 41 as nominal concentrations:

Table 41. *Daphnia magna* acute immobilization and reproduction tests: LC_{50} values (24 & 48h) and NOEC values (21d & 7d), respectively, for each bisphenol alternative tested.

Endpoint	*Daphnia*, survival		*Daphnia*, reproduction		*Daphnia*, growth	
	LC_{50}, 24h	LC_{50}, 48h	NOEC, 21d	LOEC, 21d	NOEC, 7d	LOEC, 7d
BPA						
BPZ						
BPE						
BPS-MAE	Not tested	--	--	--	--	--
BPB	Not tested	--	--	--	--	--
BPF						
BPS						
BPAF						

Red colour: values 0.015-1 mg/L,
Orange colour: values 1-10 mg/L and
Green colour: values 10-100 mg/L

Therefore, BPAF is the most toxic bisphenol for *Daphnia magna (for both survival and reproduction)*, following by BPZ, while BPA, BPE and BPF are quite less toxic but in the same order of magnitude. BPS seems to be the less toxic bisphenol for *Daphnia magna*.

Partner **SLU (SE)** performed reproductive toxicity assessment using the freshwater mollusk Greatpond snail (*Lymnaea stagnalis*) (OECD TG 243). The results are presented in Table 42 as nominal concentrations:

Table 42. *Lymnaea stagnalis* Reproduction Test: The effects of the bisphenol alternatives tested, on fecundity and mortality

Bisphenols tested		
BPA	No effects up to 0.50 mg/L (nominal)	reduced fecundity at 1.6 mg/L (nominal)
BPZ	No effects up to 0.59 mg/L (nominal)	increased mortality at 1.9 mg/L (nominal)
BPS-MAE	No effects up to 0.63 mg/L (nominal)	increased fecundity at 2.0 and 6.0 mg/L. (nominal)

Partner **SDU** (DK) also performed reproductive toxicity assessment using the freshwater mollusk Great pond snail (*Lymnaea stagnalis*) and the results are summarised in Tables 43 and 44:

Tables 43. *Lymnaea stagnalis* Reproduction Test results using BPE in different exposure concentrations

A) BPE Embryo exposure test					
Exposure (µg/L)	**Growth Reduction (%)**	**Rotation Speed Reduction**	**Heartbeat Rate (bpm)**	**Hatching Rate (%)**	**Malformations Observed**
Control (0)	0	No	83.70		No
5,000	21.5	No	39.71		Yes (& slower dev.)
10,000	36.1	Yes (Day 4)	0 (Day 6)		Yes (& slower dev.)

A) Embryo exposure test results on growth reduction, rotation speed, heartbeat rate, hatching rate and malformations. The Hatching rate is depicted in ranges:
Red color: values 0-10%, and
Green color: values 50-100%.

B) BPE Reproduction test			
Exposure (µg/L) (TWA concentr.*)	**Growth & Weight Gain**	**Mortality Rate (%)**	**Laid Egg Masses**
Control (0)	Yes		No change
4.02	Yes		No change
11.80	Yes		No change
47.36	Yes		No change
187.32	Yes		No change
872.59	Yes		No change

B) Reproduction test results on growth and weight gain, mortality rate and laid egg masses. The mortality rates are depicted in ranges:
Green color: values 0-10%
Orange color: values 10-50%
*time-weighted average (TWA) concentrations

C) BPE Transgenerational study			
Exposure (µg/L)	**F1 Embryo Development in Clear Water**	**F1 Embryo Development in BPE Exposure**	**Growth & Hatching Success**
Control (0)	No significant change	No significant change	Normal
5,000+	No significant change	Significant reduction	Lower than controls

C) Transgenerational study results on F1 embryo development and growth and hatching success

Tables 44. *Lymnaea stagnalis* Reproduction Test results using BPAP in different exposure concentrations

A) BPAP Embryo exposure test					
Exposure (µg/L)	**Growth Reduction (%) (Day 9)**	**Heartbeat Reduction**	**Development Stage Reached**	**Hatching Rate (%) (Day 18)**	**Mortality Rate (%)**
Control (0)	0	No	Normal		0
500	26.8	Yes	Normal		NS*
1,000	65.6	Yes	Did not reach veliger stage		100

A) Embryo exposure test results on growth reduction, heartbeat, developmental stage, hatching rate and mortality. The Hatching rate is depicted in ranges:
Green color for 50-100%
Orange color for 20-50%
Red color for 0-20%

B) BPAP Reproduction test						
Exposure (µg/L) (TWE concentr.*)	**Egg Mass Reduction (%)**	**BPAP 28d-EC_{50} (µg/L)**	**Feeding Behaviour (g/snail over 3 days)**	**Growth & Weight Gain**	**Survival Rate (%)**	**BPAP 28d-LC_{50} (µg/L)**
Control (0)	0		1.12	NS		
16.28	59.43		NS	NS		
36.47	NS**		NS	NS		
86.44	NS		NS	Null		
217.34	NS		NS	Null		
451.82	NS		0.013	Null		

B) Reproduction test results on egg mass reduction, EC_{50}, feeding behaviour, growth and weight, survival and LC50. The Survival rate is depicted in ranges:
Green color for 60-100%
Orange color for 20-60%
EC_{50} and LC_{50} values are depicted in ranges:
Dark red color for LC50/EC50 values 10-100 mg/L and
Light red color for LC50/EC50 values >100 mg/*NS: Not stated
*time-weighted average (TWA) concentrations
**NS: Not stated

Partner **Cefas (UK)** performed 10-day acute sediment toxicity bioassay with the marine polychaete *Arenicola marina* and the results are presented in Table 45:

Table 45. *Arenicola marina* 10-day acute sediment toxicity bioassay: EC_{50} casts and mortality concentrations (both nominal and verified) for all bisphenol alternatives tested. The EC50 values are depicted in ranges:

Chemical	EC_{50} Casts Nominal Conc. (mg/kg)	EC_{50} Casts Verified Conc. (mg/kg)	EC_{50} Mortality Nominal Conc. (mg/kg)	EC_{50} Mortality Verified Conc. (mg/kg)
BPA				
BPZ				
BPAP				
BPAF				

Red colour: values 0.5-2,

Orange colour: values 2-5 and
Green colour: values 5-10

Surviving organisms were used for the following biomarkers analysis:

Cellular Energy Allocation (CEA)

BPA and BPAP did not significantly impact the electron transport system (ETS) in *A. marina*. BPZ significantly increased ETS at 1mg/kg compared to the control group (p=0.001). Exposure to 3mg/kg sediment gave a similar ETS increase although this was not statistically significantly compared to the control samples. There was a 53 and 58% decrease in ETS at 1 and 3 mg/kg BPAF respectively.

The organisms exposed to BPA and the analogues had less carbohydrates than the control organisms. No significant difference was found at 1mg/kg in BPAP or BPZ (low treatment). At 3mg/kg (high treatment) of both BPAP and BPZ significantly decreased the levels of carbohydrates found in the test organisms. At the low concentration for BPA (3mg/kg) there was no significant difference relevant to the controls but did display a significant decrease in carbohydrates at the high concentration(10mg/kg) BPA (p=0.04). The organisms exposed to BPAF showed significant decrease in carbohydrate availability at both 1 (low) and 3 (high) mg/kg .

Overall, none of the chemicals tested showed any significant impact to protein or lipid content in *A. marina* compared to the control organisms.

Glutathione S-transferase (GST)

Surviving *A. marina* in the BPA and BPZ spiked sediment had an increased production of GST on average compared to the individuals in the control sediment. However, this increase was not significantly different from the controls.

BPAF did cause significant increase in GST production compared to the controls. BPAP also affected GST production, with a significant increase at 1mg/kg (low) and 3 mg/kg relative to the control.

Partner **Cefas (UK)** also performed the 42-day chronic toxicity test using the freshwater amphipod *Hyalella azteca,* spiking sediment with BPAF and the results are presented in Table 46:

Table 46. The 42-day chronic toxicity test on *Hyalella azteca* exposed to different BPAF concentrations: Verified concentrations and % survival are presented

Nominal Conc. of BPAF (mg/kg)	Verified Conc. (mg/kg)	Survival (%)
0	0	
0,03	<LOQ	
0,1	0,03	
0,3	0,18	
1	0,7	
3	2,58	

Green colour: Survival 83-100%

The results indicate that BPAF caused no significant effect on *Hyalella Azteca* survival. In addition, it did not result on any statistically significant effect on the number of nauplii per surviving female and final dry weight per individual.

The partner **UAVR (PT)** performed the Regeneration assay - freshwater planarian *Girardia tigrine*. The results are summarised in Table 47:

Table 47. The Regeneration assay on *G. tigrina* after several bisphenol alternatives exposure: The blastema, photoreceptor and auricle regeneration are presented along with the mortality rate

Bisphenols tested	Concentration (mg/L)	Blastema Regeneration	Photoreceptor Regeneration	Auricle Regeneration	Mortality (%)

BPA	0,04		Not reported	Not reported	
	1			Only 2 planarians regenerated	
	2,5				
	5				
BPZ	0,16				
	0,4				
	1	Regenerated initially, then died	Not evaluated	Not evaluated	
	2,5		Not evaluated	Not evaluated	
BPS-MAE	2,5				
BPAF	0,4				
	1		Not evaluated	Not evaluated	
	2,5		Not evaluated	Not evaluated	

The mortality rate is depicted in ranges:
Green colour: 0-40% and
Red colour: 40-100%.
The regeneration rate is depicted in ranges:
Green colour: increased regeneration,
Light green: not affected,
Yellow: slightly delayed,
Orange: reduced or delayed, slightly 0-40% and
Red colour: 40-100%

The partner **INERIS (FR)** was in charge of assessing the biological effects of several bisphenols in order to fill data gaps regarding their potential endocrine activities in *Daphnia magna* using recently adopted OECD test guidelines for endocrine activity, *i.e.* TG 253 - Short-term Juvenile Hormone Activity Screening Assay using Daphnia magna (JHASA).

As a first step, the toxicity of the selected bisphenols was assessed in Daphnia using OECD TG 202 and in zebrafish embryo using a refined zebrafish embryo toxicity (FET) assay (OECD TG 236 performed on tg(cyp19a1b:GFP) embryos). This first step allowed to define non-toxic concentrations of bisphenols to test in TG 253 and 250. The summarized results are the following:

BPA, BPB, BPF and BPS have been evaluated in OECD TG 202 and TG 253.

The toxicity of BPA in TG 202 was 14 mg/L. The toxicity of bisphenol substitutes ranged from 8 mg/L (BPB) to 81 mg/L (BPS).

In TG 253, BPA and BPB were inactive. BPF and BPS induced no change in the sex-ratio of juveniles (no male production). BPS induced the production of aborted eggs while BPF inhibited the molting of females and the production of the second brood.

- Zebrafish

The partner **NIB (SI)**, **BPI (EL)** and other partners as indicated below performed fish acute toxicity tests according to the OECD TG 236.

Lethal effects, calculated LC50 values for the tested bisphenols are presented in Table 48.

Table 48. The Fish Embryo Acute Toxicity (FET) Test: The nominal LC_{50} values after 96hrs of several bisphenol alternatives exposure is presented

Bisphenols tested	LC_{50} 96hrs/mg/L (SE)
	Nominal
BPA	
BPAP	
BPPH	
BPB	
BPF	
BPS	
BPAF	

The nominal EC_{50} values are presented in ranges:
Red colour: values 1-10 mg/L;
Orange colour: values 11-100 mg/L
Green colour: values >100 mg/L

The partner **UAVR (PT**) tested specific bisphenols as indicated in the table in Annex A.4 with OECD TG 236 (FET test). Preliminary results of the FET indicate that most alternatives tested were more toxic than BPA, with lower lethal concentrations (LC50) values and lower effect concentrations for malformations (both EC10 and EC50). Although the high concentrations tested may not be environmentally realistic due to the compounds' low solubility, these findings highlight the critical need for continued efforts to identify truly safer BPA alternatives. The effects of BPA and alternative compounds were also evaluated through a complementary behavioural approach and results are still being analysed.

The partner **INERIS** (FR) was in charge of assessing the biological effects of several bisphenols in order to fill data gaps regarding their potential endocrine activities in zebrafish embryo models using recently adopted OECD test guidelines for endocrine activity, i.e. TG N°250 - Detection of Endocrine Active Substances, acting through estrogen receptors, using transgenic tg(cyp19a1b:GFP) Zebrafish embrYos (EASZY assay). The summarized results are the following:

BPA and 14 bisphenols substitutes were tested using the refined OCDE TG N°236 and the TG N°250. The list of bisphenol substitutes tested is: BPA, BPB, BPE, BPC, BPC-Cl, BPF, BPS, BPS-MAE, BPS-MPE, BPZ, TCBPA, BPAF, BPAP, 4-4'ODP

9 BPA substitutes were more toxic than BPA in the refined FET assay. The LC50 for BPA was 14 mg/L and the toxicity of the substitutes range from 0.8 mg/L (TCBPA) to >100 mg/L (BPS)

At non-toxic concentrations, 14 bisphenols were assessed for their potential estrogenic activities in the EASZY assay. BPA and 13 substitutes tested elicited an estrogenic activity in the EASZY while TCBPA was inactive.

Among the active substances, nine elicited stronger estrogenic activity than BPA.

The EC50 for BPA was 0.4 mg/L while for bisphenols substitutes EC50 ranged from 0.0047 mg/L to 34 mg/L.

The toxicity and potential estrogenic activity of BPP was not evaluated yet.

As regards partner **INERIS** (FR) it was initially planned to assess the endocrine and reproductive effect of one bisphenol substitute in the OECD TG N°229 but it was not possible to perform this experiment due to "human factors" beyond partener's control. In place of performing the TGN°229, they assessed the metabolic endocrine activity of some bisphenol substitutes in a novel zebrafish embryo assay, called EMERGE (Effect of metabolic endocrine disruptors in the Gut of zebrafish embryos) which allow to evaluate the effect of chemicals on the intestinal expression of the cyp3a65 gene. The EMERGE assay is not endorsed by OECD and was developed within the Horizon EU project OBERON. In EMERGE, BPA and several bisphenols were able to induce the expression of the cyp3a65 gene in the developing intestine revealing their potential metabolic endocrine disrupting effect

The partner **SDU** (SE) was involved in the investigation of the effects of Bisphenol A (BPA)-alternatives like BPE, BPAP, and BPS-MAE and associated mixtures in the model animals, zebrafish (*Danio rerio*). They investigate possible adversity for endpoints such as heart rate, growth (larvae length), hatching rate, inflation of the posterior swimbladder chamber, eye development (eye size), development of retina structures such as retinal pigmentary layer (RPE), and inner plexiform layer (IPL), development of the brain (brain size) and associated swimming performance in a light/dark transition test (LDTT). They exposed zebrafish larvae to BPAP, BPE, and BPS-MAE and the summarized results are indicated in Table 49:

Table 49. Zebrafish exposure to BPE, BPS-MAE and BPAP: LOEC values for several endpoints are presented

Endpoint	BPE LOEC	BPS-MAE LOEC	BPAP LOEC
Larvae length decreased (mm) 5 days			
Heart rate decreased 48 hpf (bpm)		N/A	
Heart rate decreased 96 hpf (bpm)		N/A	
Heart rate increased 48 hpf (bpm)	N/A		N/A
Heart rate increased 96 hpf (bpm)	N/A		N/A
Hatching rate (%)		a	
Swimbladder inflation	a	a	a
Swimming performance changed (dark)			
Swimming performance changed (light)			
Eye size (diameter µm^2)		a	
Retinal pigmentary layer (RPE)		a	a
Inner plexiform layer (IPL)		a	a
Brain size 5 days	a	a	a

The LOEC values are presented in ranges:
Green: >1500 µg/L
Yellow: 500-1500 µg/L
Orange: 51-500 µg/L
Red: 5-50 µg/L
(a; experiments in progress, N/A not applicable)

- Alga / Aquatic plants

The partner **BPI** (EL) performed alga growth Inhibition tests and the results are shown in Table 50.

Table 50. The Alga growth inhibition test with BPF and BPS: The EC_{50} values, measured after 72 hours are presented. The nominal values are compared to the measured.

Bisphenols tested	EC_{50} 72hrs/mg/L (SE)	
	Nominal	Measured
BPF		
BPS		

The EC_{50} values are presented in ranges:
Red colour: values 10-50 mg/L
Green colour: values 50-120 mg/L

The partner **SU (SE)** performed Alga Inhibition tests with the following results as presented in Table 51 in nominal concentrations:

Table 51. The Alga growth inhibition test with several bisphenol alternatives: The nominal EC_{50} values, measured after 72 hours are presented

Bisphenols tested	Nominal EC_{50} values
BPA	
BPF	
BPS	
BPAF	

The EC_{50} values are presented in ranges:
Red colour: values 1-10 mg/L
Green colour: values 10-30 mg/L

The more toxic bisphenol was BPAF, following by BPF and BPA. As regards BPS a difference between the results derived by two partners was observed. This difference may be caused by different solvents used by each lab and the fact that the endpoints by SU was based on nominal and not measured concentration. Nevertheless, further discussion should be carried out in order to interpretate these results

The partner **IEP-NRI** performed growth inhibition test on Lemna and the results are shown in Table 52.

Table 52. The *L. stagnalis* inhibition test using bisphenol alternatives

Bisphenols tested	EC_{50} mg/L Nominal	
	frond number (f.n)	frond area (f.a)
BPZ		
BPE		
BPP		

The nominal EC_{50} values are presented in ranges:
Red colour: values 1-10 mg/L and
Green colour: values >10 mg/L

- Soil organisms

The partner **BPI (EL)** performs earthworm reproduction tests to assess the reproduction toxicity of BPZ and BPE on the earthworm *Eisenia foetida* (OECD TG 222). The LD50 values for BPZ is > 250 mg kg/dry soil, while the tests with BPE and the reproduction phase of the test with both bisphenols are ongoing.

The partner **IISPV (ES**) investigated the effects of bisphenols on soil microorganisms (OECD TG 217 and TG 216), but the tests are still ongoing.

- Amphibians

The partner **UAVR** (PT) investigated the possible effects of bisphenols in *in vitro* and *in vivo* amphibian models and the results are presented in Table 53:

Table 53. *in vitro* (A6 cell line) and *in vivo* amphibian exposure to several bisphenol alternatives: 72h- LC_{50} values (for *in vitro* protocols) and 96h LC_{50} values along with 24h EC_{50} values (*in vivo* protocols) are presented

Bisphenols tested	*In vitro* 72h- LC_{50} mg/L (A6 cell line)	*In vivo*	
		96h- LC_{50} mg/L	24h- EC_{50} mg/L for malformations

BPA			Not reported
BPS-MAE			
BPZ			Not reported
BPE			
BPAF (24h preliminary)			
BPS	NA (no activity)		
BPS-4-allyl ether	NA (no activity)		

The LC_{50} and EC_{50} values are presented in ranges:
Red colour: values 1-10 mg/L
Orange colour: values 10-50 mg/L

In vitro assays results suggest BPA to be more toxic to A6 cell line than the other tested bisphenols. For BPAF, preliminary assays using the A6 cell line indicate that this cell-based model is slightly more sensitive to BPAF than to BPA.

In vivo definitive assays show that *X. laevis* embryos are more sensitive to BPAF than to BPA (available published data and preliminary assays). Preliminary data for BPZ showed a similar toxicity trend of higher toxicity than BPA.

- In vitro / high throughput assays

The partner **BPI (EL), IEP-NRI (PL)** and **IISPV (ES)** performed microtox tests with the following results for 5, 15 and 30 min of exposure. There was not a remarkable difference in LC50 comparing the different times and the results derived by the three partners are presented in Table 54.

Table 54. The Microtox® Acute Toxicity Test using several bisphenol alternatives: The 15 min EC_{50} values. The nominal values are compared to the measured. Nominal and measured values are considered the same when they present ±20% difference. A) BPI (EL) results B) IEP-NRI (PL) results and C) IISPV (ES) results

A) Bisphenols tested	EC_{50} 15min - mg/L	
	Nominal*	Measured
BPAF		
BPF		
BPS-MPE		
BPAP		
BPP		
BPE		
BPZ		

*Nominal and measured values are considered the same when the present a maximum ±20% difference

B) Bisphenols tested	$EC_{50\ 30\ min}$ mg/L Nominal
BPAF	
BPF	
BPS-MAE	
BPS-MPE	
BPPH	

C) Bisphenols tested	EC_{50} mg/L Nominal
BPS	
BPS-MAE	
BPAP	
BPE	

In all tables the **EC_{50}** values are presented in ranges:
Red colour: values 0,5-10 mg/L
Orange colour: values 10-100 mg/L
Green colour: values >100 mg/L

The toxicity endpoints for most of the tested bisphenols were less than 10 mg/L, while only BPE, BPS, BPS-MPE, BPPH exhibited EC50 values higher than 10 mg/L. Any differences observed in the calculated values maybe be derived by the use of different solvents and will be further discussed and interpreted.

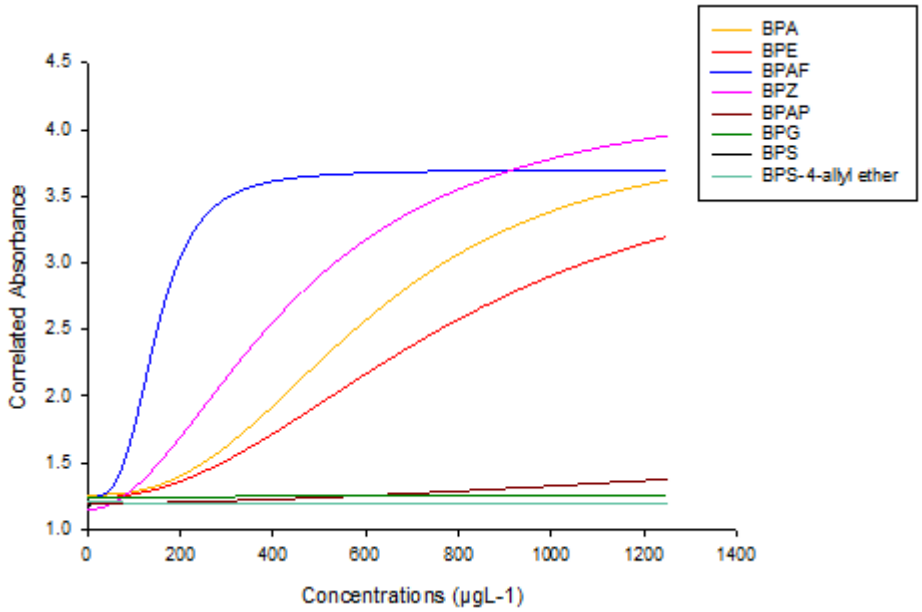
The partner **UAVR** (PT) investigated the estrogenic activity of various BPA analogues using the YES assay across a range of concentrations. The EC50 values and the dose-response curves are presented in Table 55:

Table 55. The YES assay for various bisphenol alternatives across a range of concentrations

Bisphenols tested	EC_{50} mg/L
BPA	
BPE	
BPAF	
BPZ	
BPAP	NA (no activity)
BPG	NA (no activity)
BPS	NA (no activity)
BPS-4-allyl ether	NA (no activity)

The EC_{50} values are presented in ranges:
Orange colour: values 100-150 mg/L
Green colour: values 150-850 mg/L

Fig 4. The dose- response graph between a range of bisphenol alternatives concentrations and the correlated absorbance, concerning estrogenic activity



For BPA, the graph shows a clear dose-dependent response (Figure 4), with significant estrogenic activity observed at higher concentrations. The colorimetric signal increases sharply, indicating strong activation of the estrogen receptor. Comparable to BPA, BPE also exhibits a dose-dependent increase in estrogenic activity, though with a slightly delayed response the activation becomes more prominent at higher concentrations. BPAF demonstrates a strong estrogenic activity, with a steep increase in the estrogenic activity starting at intermediate concentrations. It has the lowest EC50 and exhibits the highest activity among the tested compounds. BPZ shows notable estrogenic activity, with a dose-response curve similar to that of BPA, indicating strong receptor activation at higher concentrations.

For BPAP, no significant estrogenic activity is detected across the tested concentration range. The response remains flat, suggesting the absence of receptor activation. Similarly, BPG shows no significant estrogenic activity, with no observable colorimetric changes at the tested concentrations. BPS does not exhibit estrogenic activity within the tested concentration range, as indicated by a flat dose-response curve. BPS-4-allyl ether also shows no estrogenic activity, with a response similar to that of BPS, BPAP, and BPG.

The partner **Eawag (CH)** performed the Fish Cell Line Acute Toxicity - (OECD TG 249) to predict the acute toxicity to fish. According to this assay three EC50 values provided characterize the effect of the test chemical on metabolic activity (alamarBlue), cell membrane integrity (CFDA-AM), and lysosomal membrane integrity (Neutral Red). The lowest EC50 value obtained from the three dye measurements is used as a proxy for acute LC50 in fish. The results are presented below

Table 56. Acute toxicity of bisphenol alternative substances in RTgill-W1 assay (OECD TG 249)

Substance	Toxicity category
BPA	**II: 1 – 10 mg/L**
BPE	III: 10 – 100 mg/L
BPF	III: 10 – 100 mg/L
BPS	III: 10 – 100 mg/L
BPS-MPE*	I: ≤1 mg/L
BPS-MAE	II: 1 – 10 mg/L
BPB	II: 1 – 10 mg/L
BPAP	II: 1 – 10 mg/L
BPAF	II: 1 – 10 mg/L

BPZ	II: 1 – 10 mg/L
BPP*	I: ≤1 mg/L
bisOPP-A	I: ≤1 mg/L
TBBPA	I: ≤1 mg/L
TCBPA*	II: 1 – 10 mg/L

The toxicity category is presented per bisphenol alternative in ranges:
Red colour: values < 1 mg/L
Orange colour: values 1-10 mg/L
Green colour: values 10-100 mg/L

4. Conclusions

Human health

The selection and prioritization of bisphenol A (BPA) alternatives, including BPZ, BPE, BPS-MAE, BPP, BPAP, TCBPA, and TBBPA, with potential human health risks, were conducted through a collaborative workshop with stakeholders. A centralized approach ensured consistent test substance characterization and aligned findings. In line with the Chemical Sustainability Strategy, five human health endpoints were prioritized: 1) metabolic fate and bioactivation, 2) endocrine disruption, 3) immunotoxicity, 4) developmental toxicity, and 5) carcinogenicity. Key findings underscore the need for continued testing to avoid regrettable substitutions.

1) Metabolic Fate and Bioactivation

Radiolabeled standards were obtained for most prioritized molecules, except BPS-MAE, with purification and stability testing ongoing. BPZ's metabolism study faced challenges due to instability and solubility issues. BPE, which has better solubility, will be further evaluated to obtain reliable metabolic kinetic data for analysis.

2) Endocrine Disruption

Preliminary results from the Human H295R Steroidogenesis Assay (OECD TG 456) suggest that some BPA alternatives may be as or more potent than BPA in inducing 17β-estradiol (E2) and inhibiting testosterone (T). Additionally, these alternatives, along with BPA, may interfere with thyroid hormone regulation, inhibiting T3 transport via the MCT8 transporter and iodide uptake via the NIS transporter. These findings indicate the potential for BPA alternatives to disrupt both steroidogenesis and thyroid hormone transport, warranting further investigation into their impact on reproductive, metabolic, and developmental functions.

3) Immunotoxicity

Data indicate the need for regulatory evaluation of BPA alternatives concerning immunotoxicity. Suppression of key immune functions, such as antibody production and T-cell activation, alongside pro-inflammatory effects, suggests potential impairments in immune responses, vaccine effectiveness, and increased chronic inflammation risk. While some immune functions seem resilient, disruptions in signaling pathways and calcium homeostasis point to potential long-term immunotoxic risks, highlighting the importance of further research.

4) Developmental Toxicity

Preliminary findings show that exposure to BPA and several alternatives (BPAP, BPE, BPZ) reduce cell viability in hiPSC-derived hippocampal neurons, with BPE being more toxic at lower concentrations. In studies using neurospheres, preliminary results in DNT-IVB assays suggest that exposure to BPZ, BPAP, and BPS-MAE inhibit radial glia migration, while BPP and BPAP impaired neuronal differentiation at sub-micromolar doses. BPAP exposure disrupted oligodendrocyte differentiation, suggesting broader impacts on brain development.

Overall, these data suggest that bisphenols may present a risk to the developing nervous system and that certain alternatives may pose greater neurotoxic risks than BPA, with potential long-term effects on neurological health, cognitive function, and behavior.

5) Carcinogenicity

Preliminary in vitro testing of BPA alternatives reveals varying levels of toxicity and genotoxicity. No mutagenic effects were observed for BPE and BPP in the Ames test, but the micronucleus assay showed some genotoxicity for both. BPAP and BPS-MAE exhibited high cytotoxicity with no genotoxic effects. These alternatives appear less mutagenic than BPA but still require further evaluation using advanced assays, such as the Bhas 42 Cell Transformation Assay (OECD) and 3D hepatic cell models, which are currently in progress.

The ongoing evaluation of BPA alternatives highlights potential risks across multiple health endpoints. Some alternatives show equal or greater toxicity than BPA, emphasizing the need for further testing and risk assessment to ensure human health safety and avoid regrettable substitutions.

Environment

The key findings across species from the so far available results are highlighted below:

Aquatic invertebrates

BPAF and BPZ are the most acutely toxic (48 hrs LC50 for Daphnia <10 mg/L).

BPA, BPE, and BPF show similar toxicity (48 hrs LC50 for Daphnia LC50 ~10 mg/L) but are significantly less toxic than BPZ and BPAF.

BPS is the least toxic alternative tested (48 hrs LC50 for Daphnia LC50 ~100 mg/L), with a much higher threshold for effects.

High toxicity in *Daphnia magna* reproduction is observed for certain bisphenols (BPF, BPAF NOEC values were less than BPA).

Similarly to *Daphnia magna*, in great pond snail *Lymnaea stagnalis* BPZ caused increased mortality at low concentrations (1.9 mg/L).

BPS-MAE causes changes in the fecundity at 2-6 mg/L while BPA cause changes at slightly lower level (at 1.6 mg/L), suggesting almost similar risks as its alternatives.

BPAP and BPE exhibit effects on different parameters on *Lymnaea stagnalis (e.g.* embryo heartbeat rate and malformations, development, etc.) with BPAP being more toxic than BPE.

Aquatic invertebrates – sediment organisms

BPZ, BPAP and BPAF are less toxic than BPA as revealed by the result on mortality of Arenicola marine.

BPZ significantly increased metabolic activity (ETS) at 1 mg/kg, suggesting potential sublethal stress effects.

BPAF significantly reduced ETS and carbohydrate availability, affecting energy metabolism.

BPAP also reduced carbohydrate availability, highlighting potential metabolic toxicity.

BPAF showed no significant effects up to 2.58 mg/L on survival or reproduction, indicating lower chronic toxicity in sediment organism *Hyalella Azteca* than in *Daphnia magna.*

Regeneration Assay (Girardia tigrina, Planarian)

As regards the regeneration assay on the freshwater planarian Girardia tigrine, BPA, BPZ, BPS-MAE, BPAF were tested and they all resulted in changes in blastema, photoreceptor and auricle regeneration, as well as in % mortality in different concentrations.

Alga / Aquatic plants

BPAF is the most toxic bisphenol alternative. The next toxic alternative is BPB while BPF and BPA follow with almost similar EC50 values. No clear conclusion can be derived for BPS, since a difference between the results derived by two partners was observed and further investigation is needed.

As regards the effects on aquatic plant *Lemna minor,* BPZ was the most toxic based on the effects on frong area, while BPE and BPP were less toxic.

Zebrafish

According to the Fish Embryo Acute Toxicity (FET) Test:
TCBPA is the most toxic alternative, closely followed by BPAF, BPAP and BPPH. BPB and BPA are slightly less toxic and BPF is harmful. The least toxic alternative is BPS. Overall TCBPA>BPAF, BPAP>BPPH>BPB>BPA>BPF>BPS.

- TCBPA was inactive in the EASZY assay, while BPA appeared to be toxic (EC50<1 mg/L).

Zebrafish larvae exposure test:

- BPE is highly toxic at very low concentrations, especially affecting swimming performance and retinal development (5- 50 µg/L).
- BPS-MAE affects heart rate at low doses (<50 µg/L at 48 hpf, <1500 µg/L at 96 hpf) but has limited data on other developmental effects since the experiments are on going.
- BPAP significantly impacts hatching rate (<500 µg/L), heart rate and eye size at lower concentrations than the other tested bisphenols.

Amphibians

Behavioural Toxicity (*Xenopus laevis*)

- BPAF is the most toxic alternative (LC_{50} 96h& 24h <10 mg/L), showing strong behavioural effects. BPZ and BPA exhibit toxicity in the same order of magnitude (LC_{50} 96h <10 mg/L)

Cytotoxicity (*Xenopus* Cell Models)

- BPA, BPAF and BPS-MAE (LC_{50} 72h ~20-25 mg/L) are more toxic.
- BPE and BPZ (LC_{50} 72h ~30-40 mg/L) are the least toxic, but in the same order of magnitude as the above mentioned.
- BPS shows no toxicity.

Soil organisms

The preliminary results from the tests on earworms with BPZ and BPE showed that they are not very acutely toxic to soil organisms. However, the experiments are ongoing and the effects on reproduction will also be assessed.

In vitro / high throughput assays

Microtox acute toxicity test

- Most of the tested bisphenols show high toxicity (EC50 values < 10 mg/L) to bacterium *Vibrio fishery* (e.g. BPAF, BPF, BPAP, BPP, BPZ)
- Some other bisphenols (BPE, BPS, BPS-MAE) exhibit moderate toxicity (10 mg/L < EC50 < 100 mg/L)
- In certain cases (BPPH and BPS-MPE) differences in the toxicity were observed between the tests conducted by different partners. However, in such cases no measured values were available from all partners, with the lowest observed endpoint to be the measured one.

YES assay (investigation of estrogenic activity)

- BPAF exhibits the most estrogenic effects, showing the lowest EC_{50} (~150 mg/L) value.

- BPZ and BPA show moderate estrogenic activity (EC_{50} 400-600 mg/L).
- BPE (EC_{50} ~800 mg/L) has the weakest activity.
- BPG, BPAP, BPS, and BPS-4-allyl ether showed no estrogenic activity, suggesting they do not activate the YES assay response.

Acute Toxicity – RTgill-W1 Assay

- BPS-MPE, BPP, bisOPP-A, and TBBPA (Category I: ≤1 mg/L) are the most toxic, showing the strongest acute effects.
- BPA, BPS-MAE, BPB, BPAP, BPAF, BPZ, and TCBPA (Category II: 1–10 mg/L) exhibit moderate toxicity.
- BPE, BPF, and BPS (Category III: 10–100 mg/L) show the lowest toxicity among the tested substances.

Final Remarks:

As shown in the comprehensive testing matrix (Table 38) substances are not tested in all assays, therefore, substances not mentioned is most likely due to the lack of testing:

BPA alternatives exhibit diverse toxicological profiles, with some showing comparable or higher risks than BPA itself

BPZ and BPAF should be prioritized for regulatory assessment, given their strong effects across multiple endpoints.and species (e.g. invertebrates, alga, amphibians, microtox etc). Furthermore, BPAF is one of the most frequently detected bisphenols in aquatic compartment.

Endocrine activity data indicate potential regulatory concerns, particularly for BPS, BPS-MPE, BPS-MAE and BPF.

Metabolic and energy disruption effects (ETS, carbohydrate depletion) highlight long-term ecological risks.

Further testing is needed for BPP, which lack sufficient data for hazard characterization.

Scientific achievements

A summary of main publications and communications regarding natural toxins and BPA alternatives is provided in Table 57 and Table 58.

Table 57: Scientific outputs of T5.1. regarding natural toxins.

Title	Co-authors	Type of dissemination
A Review on aquatic toxins - Do we really know it all regarding the environmental risk posed by phytoplankton neurotoxins?	Albano Pinto, Maria João Botelho, Catarina Churro, Jana Asselman, Patrícia Pereira, Joana Luísa Pereira	Peer reviewed publications
Hazard characterization of Alternaria toxins to identify data gaps and improve risk assessment for human health	Henriqueta Louro, Ariane Vettorazzi, Adela López de Cerain, Anastasia Spyropoulou, Anita Solhaug, Anne Straumfors, Anne-Cathrin Behr, Birgit Mertens, Bojana Žegura, Christiane Kruse Fæste, Dieynaba Ndiaye, Eliana Spilioti, Elisabeth Varga, Estelle Dubreil, Eszter Borsos, Francesco Crudo, Gunnar Sundstøl Eriksen, Igor Snapkow, Jérôme Henri, Julie Sanders, Kyriaki Machera, Laurent Gaté, Ludovic Le Hegarat, Matjaž Novak, Nicola M. Smith, Solveig Krapf, Sonja Hager, Valérie Fessard,	Peer reviewed publications

	Yvonne Kohl, Maria João Silva, Hubert Dirven, Jessica Dietrich, Doris Marko	
A Short-Term Exposure to Saxitoxin Triggers a Multitude of Deleterious Effects in Daphnia magna at Environmentally Relevant Levels	Albano Pinto1,2, Inês P. E. Macário1,2, Sérgio M. Marques1,2, Maria João Botelho3,4, Jana Asselman5, Patrícia Pereira1,2, Joana L. Pereira1,2	Peer reviewed publications
Zebra-K, a kinematic analysis automated platform for assessing the sensitivity, habituation and prepulse inhibition of acoustic startle in adult zebrafish	Main author: Demetrio Raldúa Co-authors: Marija Stevanović, Niki Tagkalidou, Cristiana Roberta Multisanti, Sergi Pujol, Ouwais Aljabasini, Eva Prats, Caterina Faggio, Josep Maria Porta,	Peer reviewed publications
Hazard characterisation of the mycotoxins enniatins and beauvericin to identify data gaps and improve risk assessment for human health	Anne-Cathrin Behr, Christiane Kruse Fæste, Amaya Azqueta, Ana M. Tavares, Anastasia Spyropoulou, Anita Solhaug, Ariane Vettorazzi, Ann-Karin Olsen, Birgit Mertens, Bojana Zegura, Camille Streel, , Dieynaba Ndiaye, Eliana Spilioti, Estelle Dubreil, Franca Maria Buratti, Francesco Crudo, Gunnar Sundstøl Eriksen, Igor Snapkow, João Paulo Teixeira, Josef D. Rasinger, Julie Sanders, Kyriaki Machera, Lada Ivanova, Laurent Gaté, Ludovic Le Hegarat, Matjaz Novak, Nicola M. Smith, Sabrina Tait, Sónia Fraga, Sonja Hager, Albert Braeuning, Henriqueta Louro, Maria João Silva, Hubert Dirven, Jessica Dietrich	Peer reviewed publications
Unrevealing immunotoxic effects of bisphenol A substitutes on human macrophages, T and B lymphocytes using in vitro models	Nina Franko, Tijana Markovič, Pia Žižek, Anja Kodila, Irena Mlinarič Raščan, Marija Sollner Dolenc	Peer reviewed publications
Motor and non-motor effects of acute MPTP in adult zebrafish: insights into Parkinson's disease	Main author: Demetrio Raldúa Co-authors:Marija Stevanović, Niki Tagkalidou, Irene Romero-Alfano, Gustavo Axel Elizalde-Velázquez, Selene Elizabeth Herrera-Vázquez, Eva Prats, Cristian Gómez-Canela, Leobardo Manuel Gómez-Oliván	Peer reviewed publications
Temperature dependent sensitivity of the harpacticoid copepod Nitokra spinipes to marine algal toxins	Wenxin Liu, Ilias Semmouri, Colin Janssen, Jana Asselman	Peer reviewed publication (uploadingdata to zenodot)
Temperature and salinity affect growth and toxin content of cyanobacterium Microcystis aeruginosa (PCC 7806) in estuarine environments	Wenxin Liu, Ilias Semmouri, Colin Janssen, Jana Asselman	Peer reviewed publication (under revision)
Unlocking nature's arsenal: discovery of the Alternaria mycotoxins alterperylenol and altertoxin I as novel immunosuppressive and antiestrogenic compounds in vitro	Francesco Crudo, Vanessa Partsch, Dennis Braga, Ruzica Blažević, Judith M. Rollinger, Elisabeth Varga, Doris Marko	Peer reviewed presentation (data available on demand)
Effects of binary mixtures of cyanotoxins and xenobiotics on the growth rate of the freshwater algae Chlorella vulgaris	Carlos Pinheiro, Joana Azevedo, Alexandre Campos, Vítor Vasconcelos, Susana Loureiro	Attendance in international and national events (conference, congress, workshop, etc)
Closing data gaps on natural toxins effect on human health: Immunotoxic effects of Alternaria toxins using reporter cell lines and a 3D lung exposure model.	Solveig Krapf, Anne Straumfors, Steen Mollerup	Attendance in international and national events (conference, congress, workshop, etc)
Towards next generation risk assessment for Environmental Health: how PARC Work Package 5 is contributing	Celia Garcia Arenas, Kiara Aiello Holden, Thalia de Castelbajac, Ondrej Adamovsky, Katerina Kyriakopoulou, Jana Asselman, Gilles Riviere, Philip Marx-Stölting	Attendance in international and national events (conference, congress, workshop, etc)
Innate immune responses of Alternaria toxins in vitro: Receptor activation, inflammation induction, and signal transduction	Booshra Ahmed, Solveig Krapf, Anne Straumfors, Paal Graff, Steen Mollerup.	Attendance in international and national events

		(conference, congress, workshop, etc)
Closing data gaps on natural toxins effect on human health: Immunotoxic effects of Alternaria toxins using a co-culture lung exposure model	Solveig Krapf, Anne Straumfors, Steen Mollerup National Institute of Occupational Health (STAMI) Gydas vei 8, 0333 Oslo, Norway"	Attendance in international and national events (conference, congress, workshop, etc)
Immunomodulating effects of Alternaria toxins activation on the toll like receptor – NFkB/AP-1 signalling pathway	Solveig Krapf, Anne Straumfors, Steen Mollerup.	Attendance in international and national events (conference, congress, workshop, etc): poster
Investigating the genotoxic effects of the Alternaria toxin Tenuazonic Acid in human liver cells	Beatriz Guerreiro, Célia Ventura, Henriqueta Louro, Maria João Silva	Attendance in international and national events (conference, congress, workshop, etc): Poster
METABOLISM OF ENNIATIN B IN PRIMARY MOUSE, RAT AND HUMAN HEPATOCYTES	Estelle DUBREIL, Lada IVANOVA, Charlène GENDRE, Mariam MADJHOUB, Valérie FESSARD, Ludovic LE HEGARAT, Christiane K. FAESTE, Jérôme HENRI	Attendance in international and national events (conference, congress, workshop, etc): Poster
Steroidogenesis assay (OECD TG 456) to fill data gap on BPA alternatives and on the natural mycotoxins Enniatins and Beuvericin: preliminary results from two PARC projects	Lucia Coppola, Gabriele Lori, Elena Bossù, Livia Manna, Daniele Sadutto, Sabrina Tait	Attendance in international and national events (conference, congress, workshop, etc): Poster
Contribution to the characterisation of Alternaria toxins' genotoxicity in human liver cells	B. Guerreiro, C. Ventura, H. Louro, M. J. Silva	Attendance in international and national events (conference, congress, workshop, etc): Poster
Influence of Temperature on Acute and Chronic Toxicity of Marine Algal Toxins — A Case Study with Copepod Nitokra spinipes	Wenxin Liu, Ilias Semmouri, Colin Janssen, Jana Asselman	Attendance in international and national events (conference, congress, workshop, etc): Poster
Acute Toxicity of Harmful Algae on Marine Zooplankton in the Context of Climate Change	Wenxin Liu1, Suzanne Bulckaert, Ilias Semmouri, Colin Janssen, Jana Asselman	Attendance in international and national events (conference, congress, workshop, etc): Poster
Genotoxicity assessment of enniatins and Alternaria toxins with the in vitro micronucleus assay and the SOS/umu test	Streel Camille, Vettorazzi Ariane, Sanders Julie, Anthonissen Roel, Azqueta Amaya and Mertens Birgit	Attendance in international and national events (conference, congress, workshop, etc): Poster
Closing regulatory data gaps on the genotoxicity and non-genotoxic carcinogenesis of natural toxins and bisphenols	Maria João Silva, Kiara Aiello-Holden, Doris Marko, Jessica Dietrich, Henriqueta Louro, Hubert Dirven	Attendance in international and national events (conference, congress, workshop, etc) presentation
Genotoxicity assessment and potency ranking of enniatins and Alternaria toxins with the in vitro micronucleus assay	Streel Camille, Sanders Julie, Anthonissen Roel and Mertens Birgit	Attendance in international and

		national events (conference, congress, workshop, etc)
INTERSPECIES VARIATIONS IN THE METABOLISM OF ALTENUENE AND TENTOXIN IN PRIMARY HEPATOCYTES	Eszter Borsos, Charlène Gendre, Elisabeth Varga, Estelle Dubreil, Jérôme Henri, Ludovic Le Hegarat, Doris Marko	Attendance in international and national events (conference, congress, workshop, etc) - poster
Mycotoxin Metabolism Matters: Unlocking the Immunotoxicity of Enniatins and Beauvericin	Dino Grgic, Ibrahim Elesh, Vanessa Partsch, Francesco Crudo, Sonja Hager, Doris Marko	Attendance in international and national events (conference, congress, workshop, etc) - to be decided (poster or presentation)
Assessing the effects of phycotoxin mixtures on marine zooplankton: insights from copepod responses at different life stages	Ilias Semmouri, Luca Deroma, Colin R. Janssen, Jana Asselman	Attendance in international and national events (conference, congress, workshop, etc)

Table 58: Scientific outputs of T5.1. regarding BPA alternatives

Title	Co-authors	Type of dissemination
Unrevealing immunotoxic effects of bisphenol A substitutes on human macrophages, T and B lymphocytes using in vitro models	Nina Franko, Tijana Markovič, Pia Žižek, Anja Kodila, Irena Mlinarič Raščan, Marija Sollner Dolenc	Peer reviewed publications
Temperature and salinity affect growth and toxin production of cyanobacterium Microcystis aeruginosa (PCC 7806) in estuarine environments	Wenxin Liu, Ilias Semmouri, Colin Janssen, Jana Asselman	Peer reviewed publications
Endocrine disruption, adverse outcomes and occurrence of newly emerging BPA substitutes	Nina Franko, Anja Kodila, Marija Sollner Dolenc	Peer reviewed publications
Endocrine disrupting toxicity of bisphenol A and its analogs: impact on the neuro-immune system	Erica Buoso, Mirco Masi, Roberta Limosani, Chiara Oliviero, Martina Iulini, Francesca Passoni, Marco Racchi and Emanuela Corsini	Peer reviewed publications
Comprehensive Toxicity Scoring of 26 BPA Alternatives Based on Eight Cell-Based Bioassays and Abiotic CYP Oxidation Test	V. Srebny, L. Henneberger, J.Huchthausen, M. König, S. Mälzer, and B. I. Escher	Peer reviewed publications
Which in vitro liver model suits best for predictive toxicological research: A comparison	Yvonne Kohl, Gizem Erdogan, Sylvia Wagner	Attendance in international and national events (conference, congress, workshop, etc): Poster
Temperature and salinity affect growth and toxin production of estuarine cyanobacterium Microcystis aeruginosa	Wenxin Liu, Ilias Semmouri, Colin Janssen, Jana Asselman	Attendance in international and national events (conference, congress, workshop, etc) - presentation
Studio in vitro del bisfenolo A e dei suoi analoghi nella regolazione della risposta immunitaria	Francesca Carlotta Passoni, Martina Iulini, Valeria Bettinsoli, Valentina Galbiati, Marina Marinovich, Emanuela Corsini	Attendance in international and national events (conference, congress, workshop, etc)
Linking chemical disruption of molting processes to survival of crustaceans	Knut Erik Tollefsen, Bjørn Henrik Hansen, Simon Schmid, You Song, Celine Vågå, Li Xie.	Attendance in international and national events (conference, congress, workshop, etc)

Is it safer? Evaluating the toxicity of Bisphenol A Alternatives in Zebrafish Embryos	Maria S. Costa, Marta S. Monteiro, Susana Loureiro, Inês Domingues	Attendance in international and national events (conference, congress, workshop, etc)
In vitro effects of BPA and its analogues on antibody production	Martina Iulini	Attendance in international and national events (conference, congress, workshop, etc): Presentation
In vitro effects of bisphenol A analogues on human B and T lymphocytes activation	F. C. Passoni, A. Maddalon, V. Bettinsoli, V. Galbiati, and E. Corsini. Università degli Studi di Milano, Milan, Italy.	Attendance in international and national events (conference, congress, workshop, etc)
Impact of endocrine disruption on the number of hair cells in neuromasts of the lateral line organ of zebrafish larvae (Danio rerio)	Ellen Vandeputte Evelyn Stinckens, Lucia Vergauwen, Dries Knapen	Attendance in international and national events (conference, congress, workshop, etc)
Impact of endocrine disruption on the number of hair cells in neuromasts of the lateral line organ of zebrafish larvae	Ellen Vandeputte, Evelyn Stinckens, Lucia Vergauwen, Dries Knapen	Attendance in international and national events (conference, congress, workshop, etc): poster
Impact of endocrine disruption on the number of hair cells in neuromasts of the lateral line organ of zebrafish larvae	Ellen Vandeputte, Evelyn Stinckens, Lucia Vergauwen, Dries Knapen	Attendance in international and national events (conference, congress, workshop, etc) presentation
IDO-1 activation is a key event, driving immunotoxicity	Lucia Parrakova, Oriol Ruiz Catalan, Pablo Monfort-Lanzas, Dietmar Fuchs, Johanna M Gostner	Attendance in international and national events (conference, congress, workshop, etc)
ENDOCRINE DISRUPTION IN DAPHNIA MAGNA: FROM MOLECULAR TO POPULATION LEVELS	MADALENA VIEIRA , MARIA PAVLAKI , SUSANA LOUREIRO	Attendance in international and national events (conference, congress, workshop, etc)
Effects of newly emerging bisphenols on T-cell activation	Nina Franko, Marco Bertolli, Anja Kodila, Emanuela Corsini and Marija Sollner Dolenc	Attendance in international and national events (conference, congress, workshop, etc)
Effects of Bisphenols to Amphibians and Fish: An Integrative Approach	Barreto M., Costa M.S., Vilarinho I., Marques B., Quintaneiro C., Monteiro M.S., Domingues I., Oliveira M. and Lopes I.	Attendance in international and national events (conference, congress, workshop, etc)
Ecotoxicological Evaluation of Bisphenol A and alternatives: A Comprehensive in silico Modelling Approach	Liadys Mora Lagares and Marjan Vračko	Attendance in international and national events (conference, congress, workshop, etc)
Contribution to the hazard assessment of substances alternative to bisphenol A: genotoxic and carcinogenic effects in mammalian cells	Pereira M., Tavares A., Louro, H., Silva M.J.	Attendance in international and national events (conference, congress, workshop, etc): Poster
Comparative Toxicity Assessment of Bisphenol A and Its Analogues (BPAF and BPZ) Using Zebrafish (Danio rerio) Embryos and Larvae as Model Organisms	Maria S. Costa, Inês Vilarinho, Marta S. Monteiro, Isabel Lopes and Inês Domingues	Attendance in international and national events (conference, congress, workshop, etc) - poster
Closing regulatory data gaps on the genotoxicity and non-genotoxic carcinogenesis of natural toxins and bisphenols	Maria João Silva, Kiara Aiello-Holden, Doris Marko, Jessica Dietrich, Henriqueta Louro, Hubert Dirven	Attendance in international and national events (conference, congress, workshop, etc) presentation
BPA to Z: Harnessing Bioanalytical Tools for the Development of Hazard-Free Chemicals	"V. Srebny, L. Henneberger, J.Huchthausen, M. König, S. Mälzer, and B. I. Escher Department Cell Toxicology,	Attendance in international and national events (conference, congress, workshop, etc): Presentation

	Helmholtz Centre for Environmental Research – UFZ	
Assessing the effects of phycotoxin mixtures on marine zooplankton: insights from copepod responses at different life stages	Wenxin Liu, Ilias Semmouri, Luca Deroma, Colin Janssen, Jana Asselman	Attendance in international and national events (conference, congress, workshop, etc) - poster
Advancing the understanding of the life-stage specific impact of thyroid hormone system disruption in the zebrafish embryo model	Imke Van Dingenen, Lucia Vergauwen, Dries Knapen	Attendance in international and national events (conference, congress, workshop, etc): poster

Interactions with regulators

Ongoing monthly meetings with the Task/Activity Leaders 5.1.1, Project Managers, Endpoint leaders and partners. Our project reviewers are mostly experts from EFSA & ECHA and to have a better overview and understanding of our work they are joining our monthly meetings. The participants are as follow:

Regulatory Agencies: ECHA, EFSA, EEA are welcome to join. So far, only experts from EFSA/ECHA have joined our monthly meetings. Project Managers of P5.1.1.a Natural toxins have been invited to the EFSA focus group on Alternaria, to share preliminary results. A5.1.1 Activity Leaders [NIPH and INSA]

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Annex

A.0 Participants

A.01 Participants: Toxins human health

Project Manager Enniatins :

BfR (Germany): Anne-Cathrin Behr. Jessica Dietrich from 2022 until2024

Project Manager *Alternaria* toxins:

UNIVIE (Austria): Doris.Marko

Partners List (besides management):

ANSES (France): Ludovic Lehegarat

INRS (France): Laurent Gate, Dieynaba Ndiaye

Sciensano (Belgium): Julie Sanders, Camille Streel

NIPH (Norway): Hubert Dirven, Igor Snapkow, Nicola Margareta Smith

IMR (Norway): Josef Rasinger

STAMI (Norway): Steen Mollerup, Solveig Krapf

INSA (Portugal): Henriqueta Louro, Maria Joao Silva,

NIB (Slovenia): Bojana Zegura, Matjaz Novak

UNAV (Spain): A. Vettora

BPI (Greece): E. Spilioti

Fraunhofer-IBMT (Germany): Yvonne Kohl

ISS (Italy): Sabrina Tait

NVI (Norway): Gunnar Eriksen, Christiane Faste

TUB (Germany): Roderich Suessmuth

A.02 Participants: Toxins environment

Project Manager

UGent (Belgium): Jana Asselman. Researchers: Eveline Diopere

Deputy Project Manager

SLU (Sweden): Stefan Örn. Researcher: Gunnar Carlsson

Partners List (besides management):

UAVR (Portugal): Susana Loureiro
UoB (United Kingdom): Pu Xia

A.03 Participants: BPA alternatives human health

Project Manager

BfR (Germany): Kiara Aiello

Endpoint Leaders/Project Managers

1. Endocrine Disruptors:

ISS (Italy): Sabrina Tait
INRAE (FR): Catherine Viguié

2.Developmental Neurotoxicity

CNRS (FR): Sakina Mhaouty-Kodja. Researcher: Lydie Naule

3. Immunotoxicity:

UMIL (Italy): Emanuela Corsini. Researcher: Martina Lulini

4. Carcinogenicity:

INSA (Portugal) Maria Joao Silva. Researcher: Ramos Carolina

5. Metabolism:

INRAE (France): Daniel Zalko

ISS (Italy): Buratti Franca. Emanuela Testai from 2022 until 2025.

Partners List (besides management):

ISS (Italy): Francesca Marcon (Carcinogenicity). Simonetta Palleschi (Immunotox)

TTL (Finland) Jonna Weisell, Kukka Aimonen (Carcinogenicity)
ULFFA (Slovenia) Marija Sollner Dolenc, Nina Franko (Immunotoxicity)
MUI (Austria) Johanna Gostner
ISCIII (Spain) Ana Isabel Cañas Portilla, Antonio De La Vieja Escolar (ED)
INRS (France) Laurent Gate, Sophie Ndaw, Yves Guichard (Carcinogenicity)
IBMT Fraunhofer (Germany) Sylvia Wagner, Yvonne Kohl Carcinogenicity)
LIH (Luxembourg): Archibold Mposhi (joined Febrary 2024); Jonathan Turner.
Nathalie Grova from 2022 until 15th March 2024) (DNT)
NILU (Norway) Naouale El Yamani Carcinogenicity)

A.04 Participants: BPA alternatives environment

Project Manager

BPI (Greece): Katerina Kyriakopoulou (for Data Gaps section of the porject)

Partners List (besides management):

MU (Czechia)

SLU (Sweden) IISPV (Spain)

SDU (Denmark)
INERIS (France)
NIB (Slovenia)
IEP-NRI (Poland)
UU (Sweden)
UG-PL (Poland)
UAVR (Portugal)
EAWAG (Switzerland)
NIVA (Norway)
UFZ (Germany)
INRAE (France)
SU (Sweden)
Cefas-Defra (UK)

A.1 Methods: Toxins human health

Genotoxicity and mutagenicity

The **Ames assay** for ENNs was performed according to the OECD TG 471 (OECD 2020) in the *Salmonella* Typhimurium strains TA98, TA1537, TA1535 and TA100 as well as in the *Escherichia coli* strains WP2. The Test has started recently in January 2025 with range finding tests [BfR (DE)]. Due to the high procurement costs, a simplified Ames assay was conducted for the *Alternaria* toxins [NIB (SI)]. The following modifications were made to the guideline for this purpose: In the assay, five *Salmonella* Typhimurium strains were used (TA97a, TA98, TA100, TA102 and TA1535) without and with metabolic activation system (post-mitochondrial rat liver fractions S9, 10%, simulating metabolic activation). The highest soluble concentration of each toxin in DMSO (data provided within the consortium) was used as the highest treatment concentration in the AMES assay.

The ***in vitro* mammalian Micronucleus assay** in TK6 cells were performed according to the OECD TG 487 using cytochalasin B as cytokinesis blocker (OECD 2023a). TK6 cell being metabolically incompetent, all the selected mycotoxins were tested for 24h without metabolic activation and 3h with metabolic activation (S9 fraction). In case of a negative result in both tests, the mycotoxins were also tested for 3h without metabolic activation.

For the **micronucleus test** in HepG2 cells, cells were seeded at 2×10^5 cells per well in 6-well plates, with two replicates per concentration and controls, for 24h at 37ºC and 5% CO2. HepG2 cells were exposed for 3h (short treatment) to 2 mL of 1, 5, 10, 20 and 30 µM of AME; 1, 10, 50, 75 and 100 µM of TeA, MMC at 0.15 µg/mL (positive control) or culture medium (negative control). For each well, two slides were prepared, dried at room temperature, stained with a 4% Giemsa solution, mounted, coded and analyzed in an optical microscope. MN frequencies were analyzed in 1000 BNC from each replicate. Additionally, 1000 cells per culture were scored to assess the Cytokinesis Block Proliferation Index (CBPI), and Replication Index (RI).

For **γH2Ax assay**, undifferentiated HepaRG cells were plated in 96-well plates at a density of 9.000 cells per well and cultured according to previously established protocols (Luckert et al., 2018). Differentiated HepaRG cells were exposed for 24h to various concentrations of mycotoxins in the differentiation medium containing 1.7% DMSO, Doxorubicin (1 µM) served as a positive control and untreated cells as a negative control.

The **SOS/umu** test was performed according to the method used in Alonso-Jauregui et al. (2021) with some modifications. In each test performed, negative and positive controls were included, DMSO (or water for FB1) was used as solvent control (negative control), and 4-nitroquinoline-N-oxide (4NQO) and 2-aminoanthracene (2-AA) were used as positive controls in the absence and presence of S9 mix, respectively.

Endocrine effects

For the **ER SSTA**, the hERα-HeLa9903 cell line was purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB1318-HeLa9903, JCRB, Osaka Japan). The cell line can be used to identify human estrogen receptor α (hERα) agonists and antagonists, as detailed in the OECD test guideline 455 for the testing of chemicals (OECD 2021).

For the **AR SSTA**, the AR-EcoScreen™ cell-based reporter assay was used, expressing the human AR and an AR inducible luciferase reporter gene resulting in androgen-dependent changes in luminescence. It can be used to identify human androgen receptor (hAR) agonists and antagonists, as detailed in the OECD test guideline 458 for the testing of chemicals (OECD 2023b).

The **steroidogenesis assay** was performed according to the OECD TG 456 implying the human adreno-carcinoma cell line NCI-H295R cells from ATCC (OECD 2023c). A commercial ELISA kit was used to assess E2 levels, diluting samples 1:50. So far, the hormone levels in two out of three biological replicas from ENN B and B1 treated cells were assessed. Hormone interference test was performed for these compounds according to TG 456 protocol; at the maximum concentration tested, the compounds interfered < 20% with hormone assessment as requested. The steroidogenesis assay for *Alternaria* toxins has recently started with finding an appropriate hormone analysis method by performing the matrix and chemical interference tests of several ELISA test kits [BfR (DE)].

Immunotoxic effects

The **NF-κB reporter gene assay** was performed in 96-well plates, with 100 µL of each compound (ENNs and BEA in a concentration range of 0.1–5 µM) pipetted into the wells. THP1-Lucia™ monocytes were centrifuged at 140 × g for 2 min at room temperature. After discarding the supernatant, the cell pellet was resuspended to achieve a final concentration of 100,000 cells/100 µL. 100 µL of the cell suspension was added to each well containing the pre-diluted test substances. The plate was gently shaken at 500 rpm for 1 minute and incubated for 2h at 37°C in a 5% CO_2 atmosphere. LPS (10 ng/mL) was then added to all wells, excluding the medium and solvent controls, to induce NF-κB activation. The plate was shaken again for 1 min at 500 rpm and incubated for an additional 18h to allow for luciferase protein expression and secretion. Following incubation, the plate was centrifuged at 140 × g for 2 min and 20 µL of the supernatant was transferred to a white 96-well plate. Subsequently 50 µL of the QUANTI-Luc™ reagent (InvivoGen, USA) was added and luminescence was directly measured with the Cytation 3 Cell Imaging Multi-Mode Reader (Biotek®, Winooski/VT, USA). Results were expressed as mean ± SD of at least three biological replicates and normalized to the LPS control (10 ng/mL; set to 100%).

For the **TLR reporter assay**, human embryonic kidney (HEK) 293 reporter cells for TLR2 and TLR4 as well as parental cell line HEK-null1 (Invivogen, France). The reporter cells specifically expressed TLR 2 or TLR4-inducible reporter genes with an intact NF-κB pathway encoding secreted embryonic alkaline phosphatase (SEAP) through activation of NF-κB. The cells were maintained in Dulbecco's modified Eagle medium [DMEM] -Glutamax and high glucose supplemented with 10% fetal bovine serum and manufacturer-recommended HEK Blue selection antibiotics. Cells were subcultured every 2-3 days to maintain confluency below 80% and passage below 20, they were kept in humidified incubators at 37°C and 5% CO2. HEK-TLR2 and HEK-TLR4 cells were seeded at ~50,000 cells/well in flat bottomed 96 well trays (Sarstedt) and incubated for 24h before exposed to either control media or different concentrations of mycotoxins produced by Alternaria species (AOH (>30µM), AME (>10µM), TeA(>100µM), ATX-1(>20µM), ALT(>0µM), and TEN(>10µM)) plus/minus LTA (100 ng/ml) TLR2 cells or LPS (10 ng/ml) TLR4 cells. After 24h of incubation 10 µl of cell supernatant was transferred to a new plate and supplemented with 90 µl freshly made Quanti-Blue solution (invivogen, France). Samples were incubated for 3h absorbance was read at 649 nm, using a SpectraMax i3 equipped with SoftMax Pro 6.3.1 software (Molecular Devices LLC, San Jose CA, USA). As a negative control sample growth media was added. For positive control Lipoteichoic acid (LTA; invivogen France) was used for TLR2, ultrapure lipopolysaccharide (LPS; invivogen, France) was used for TLR4).

Additional assays and in silico analysis

In silico prediction for biotransformation and toxicity were performed for ENNA, ENNA1, ENNB and ENNB1 and BEA. Prediction of biotransformation products were performed by Meteor Nexus version 3.2.0 (KB 1.0.0, Lhasa Limited) using the method Site of Metabolism Scoring, and Derek Nexus (v.6.3.0) was used as a Structure-Activity Relationship (SAR) tool for toxicity predictions.

To assess the **intrinsic clearance** for ENNs and Alternaria toxins, human (20 Caucasian donor mixed gender pooled: 10 men and 10 women), rat (Male Sprague-Dawley) and mice (Male CD-1) hepatocytes were used. Concentrations used for clearance were based on cytotoxicity experiments. Cells were thawed and suspended in Williams E medium (Eurobio, Les Ulis, France), supplemented with an additive for hepatocyte culture medium purchase from Wepredic. Cell density was adjusted at 1.106 cells/mL and supposed to be constant during the treatment, based on cytotoxicity experiments. In glass tube suspended hepatocytes were incubated with ENNA (1 µM), ENNA1 (1 µM), ENNB (1 µM), ENNB1 (1 µM), ALT (10 µM) and TEN (10 µM), at 37°C with 5% CO2 on a microplate agitator. At 0, 0.5, 1, 1.5, 2 and 4h, cells were put on ice to stop metabolism and centrifuged. The supernatants were transferred in amber glass vial and stock at -80 °C until analysis.

A.2 Methods: Toxins environment

Acute and chronic toxicity experiments were executed with the benthic estuarine copepod species *N. spinipes*, following the guidelines described in the International Organization for Standardization method (ISO) 14669 for acute tests, larval development was assessed based on ISO 18220 (ISO, 2016) and following the guidance document ENV/JM/MONO(2014)17 described by Organization for Economic Co-operation and Development (OECD) (OECD, 2014) for reproduction studies in chronic tests, with an average duration of 33 to 36 days as specified in the guideline and dependent on the number of broods of the control group. Specifically, the toxicity of the following four natural aquatic toxins was evaluated chronically: domoic acid (DA), yessotoxin (YTX), saxitoxin (STX), and microcystin-LR (MC-LR) were examined at 18, 20, and 22°C with environmentally relevant concentrations. Acute tests for DA and YTX were executed with both adults and nauplii and evaluated mortality. Additionally, the effects of the aquatic toxin MC-LR was also evaluated through the dosing of a MC-LR producing cyanobacteria on two copepod species, i.e. *N. Spinipes* and *A. Tonsa* and effects on mortality and swimming behaviour. The concentration of MC-LR in the producing cyanobacteria was verified through ELISA.

Additional mixtures studies are currently ongoing. Chronic experiments with *Lymnaea stagnalis* followed the OECD test guideline 243, *Lymnaea stagnalis* Reproduction Test. Here, we evaluted effects of microcystin, 0.5 to 50 µg/L, Zearalenon, 0.030 to 0.50 mg/L. Mixture study on Deoxynivalenol (Don), Zearalenone (Zen), Enniatin A1 (EnnA1) and Enniatin B1 (EnnB1) based on environmental relevant levels is currently ongoing for this organisms. Finally, we investigated the combined toxicity of cyanotoxins and anthropogenic contaminants on the growth of *Chlorella vulgaris*, focusing on both single and binary mixture exposures. Here, all concentrations were analytically verified using HPLC-MS. The test substances included cadmium chloride ($CdCl_2$), terbuthylazine (TBA), microcystin-LR (MC-LR), and cylindrospermopsin (CYN). These compounds were selected due to their environmental relevance and potential interactive effects. The freshwater microalgae C. vulgaris served as the test organism, cultivated under controlled laboratory conditions to ensure axenic growth. To assess toxicity, growth inhibition was used as the primary endpoint. This was done based on OECD 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test (Chlorella vulgaris). Specific measurements included the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC), along with EC50 values, which indicate the concentration required to reduce growth by 50%. Additionally, interactions between chemicals in binary mixtures were analyzed to determine whether they exhibited synergistic, additive, or antagonistic effects.

A.3 Methods: BPA alternatives human health

Metabolism bioactivation routes and kinetics

Chemicals, materials, and reagents

Bisphenols were purchased from BCP-Instruments-Chiron and dissolved in dimethyl sulfoxide (DMSO). Radio-labelled molecules were purchased from RC TRITEC (Teufen, Switzerland). Cell culture plastics were obtained from BD Bioscience (France), except for flasks and 96-well transparent plates which were purchased from TPP (Dutscher, Bernolsheim, France). Dimethyl sulfoxide (DMSO), penicillin, streptomycin, and insulin were purchased from Sigma-Aldrich Merck (Saint-Quentin Fallavier, France). Gibco Williams'E medium with and without phenol red, and Prestoblue cell viability reagent were purchased from Thermo Fisher Scientific (Illkirch, France). DPBS was purchased from PanBiotech (Aidenbach, Germany). Hydrocortisone was purchased from SERB (Paris, France).

Flo-Scint II and Ultima-Gold liquid scintillation cocktails were purchased from PerkinElmer Sciences (Courtaboeuf, France). HPLC-grade solvents (ethanol, acetonitrile) were purchased from Revvity (Perkin Elmer, Courtaboeuf, France).

Ultrapure water produced with the Milli-Q system (Millipore, Saint-Quentin-en-Yvelines, France) was used for preparing HPLC mobile phases.

[^{3}H]-bisphenol A ([^{3}H]-BPA; specific activity: 185 GBq/mmol) and [^{3}H]-bisphenol S ([^{3}H]-BPS; specific activity: 140.6 GBq/mmol) were purchased from Moravek (Brea, CA, USA). [^{3}H]-bisphenol B ([^{3}H]-BPB; specific activity: 1969.51 GBq/mmol), [^{3}H]-bisphenol P ([^{3}H]-BPP; specific activity: 2229.25 GBq/mmol), [^{3}H]-bisphenol Z ([^{3}H]-BPZ; specific activity: 2345.8 GBq/mmol) and [^{3}H]-pergafast 201 ([^{3}H]-PF201; specific activity: 2704.7 GBq/mmol) were purchased from RC TRITEC (Teufen, Switzerland). They were further purified by radio-HPLC to reach a radiopurity greater than 95%.

^{1}H-NMR analysis of non-labelled standards

All compounds were dissolved in 200 µl of deuterated methanol, except PF-201, which was dissolved in 200 µl of deuterated acetonitrile. Solutions were transferred into 3 mm NMR tubes. ^{1}H-NMR spectra were obtained on a Bruker Avance III HD NMR spectra operating at 600.13 MHz for ^{1}H resonance frequency using an inverse detection 5 mm ^{1}H-^{13}C-^{15}N-^{31}P cryoprobe attached to a Cryoplatform (the preamplifier unit). ^{1}H-NMR spectra were acquired at 300 K using a standard sequence. A total of 32 scans were collected into 65536 data points using a spectral width of 20 ppm, a relaxation delay of 5 s, and an acquisition time of 2.72 s. Prior to Fourier transformation, an exponential line broadening function of 0.3 Hz was applied to free induction decays. Spectra were manually phased, referenced to methanol signal (d 3.31 ppm) or acetonitrile signal (d 1.94 ppm) and the baseline was corrected using Topspin 3.6.4 software (Bruker, Karlsruhe, Germany).

HepaRG cells culture conditions

In this study, the metabolically competent cell line HepaRG was used. A preliminary step of the differentiation of HepaRG cells was carried out upstream. Briefly, cells were thawed and put into proliferation stage for two weeks at 37°C, in growth medium (Williams'E containing phenol red, supplemented with 10% FCS, 1% penicillin/streptomycin, 0.1% insulin, 0.1% glutamine, and 0.1% hydrocortisone). Then, cells were allowed to differentiate for two weeks, in Williams'E containing phenol red, supplemented with 10% FCS, 1% penicillin/streptomycin, 0.1% insulin, 0.1% glutamine, and 0.1% hydrocortisone, and containing 2% DMSO.

Viability test in HepaRG cells

At the end of the differentiation period, HepaRG cells were trypsinized and seeded at 72,000 cells per well and left to incubate for 48h before chemical exposure. Solvent content was used at 0.1% v/v in the culture medium. Cells were exposed to the range of selected chemicals for 24 hours at 37°C. Cell viability was then determined in living cells as follows: the culture medium was removed and replaced with Prestoblue® diluted in the culture medium. After 2 hours of incubation at 37°C, the fluorescence emitted was quantified by spectrophotometry (excitation wavelength = 535 nm, emission wavelength = 615 nm, Tecan plate reader Infinite 200). Cell viability was expressed as a percentage of the negative control.

Mass balance studies in HepaRG cells

After differentiation, HepaRG cells were trypsinized, seeded in 24-well transparent flat-bottom plates at 480,000 cells per well, and incubated for 48h prior to chemical exposure. Mass balance assays have been started for BPA, BPB, BPP, BPS, BPZ, PF-201. These assays were carried out using [^{3}H]-labelled molecules, at 6 non-cytotoxic

concentrations. When necessary (higher concentrations), [^{3}H]-labelled molecules were supplemented with the equivalent non-labelled molecule (Chiron standards).

Cells were exposed to the radio-labelled compounds for 24 hrs at 37°C, in a final volume of 500 µL culture medium, with 0.1% v/v of DMSO. To detect potential degradation under cell culture conditions, radiolabelled compounds were incubated in medium without cells.

After 24-hr exposure, culture media were individually collected in 1.5mL micro tubes. Triplicates of 10µL samples, for each assayed concentration, were mixed with scintillation solution for radioactivity determination. The remainder of these samples was immediately stored at -80°C for further radio-HPLC analyses. Cells were then individually washed with 1mL cold (4°C) PBS. The PBS was then collected in 1.5mL micro tubes. Triplicates of 50µL of the washing solutions were collected for radioactivity measurements, the rest being immediately stored at -80°C. 500µL of a water-acetonitrile mix 50/50 (v/v) was added in each well. Cells were scraped with a cell scrapper and each well content was individually collected. This step was repeated once. Triplicates of 50 µL were sampled for radioactivity determination, the remainder being stored at -80°C.

Radioactivity measurements were carried out using a Tri-Carb 2910TR (PerkinElmer) liquid scintillation analyser using Ultima Gold® as scintillation cocktail. Sample quenching was compensated by the use of quench curves and external standardization.

Radio-HPLC metabolic profiling

Culture media were individually analysed by reversed-phase high-performance liquid chromatography coupled with radio-detection (R-HPLC) for chromatography profiling and quantification. Analyses were performed on an Ultimate-3000 system (Thermo Fisher Scientific) coupled with a flow scintillation analyser Flo-One Radiomatic® 610TR.

Further detailed results: Metabolism bioactivation routes and kinetics:

Initiation of biotransformation studies in HepaRG cells (INRAE, FR)

Based on the determination of substance-specific highest non-cytotoxic concentrations in HepaRG cells, INRAE derived for each compound of interest a range of 6 concentrations for metabolic studies (Table A1) which were initiated in HepaRG cells. Work is in progress, with a similar approach implemented for each molecule. First, a 24-hr mass balance is to be established by summing the radioactivity present in incubation media, cell content and cells/plates washings. Mass balance will be considered acceptable when radioactivity recovery averages 100 ± 10% of the radioactivity put in incubations. If mass balance highlight incomplete radioactivity recovery, additional work is carried out to examine whether molecules (or their metabolites) are unstable, adhere to plasticware, or produce adducts with cell macromolecules. In a second step, radio-HPLC metabolic profiling will be carried out on incubation media. To this end, INRAE currently develops substance-specific analytical conditions, with the aim to separate (and quantify) all major metabolites for the prioritized molecules. These analyses will be extended to cellular content extracts when applicable (e.g. when the intra-cellular radioactivity content allows so). All experiments are to be carried out in triplicate for each of the 6 non-cytotoxic concentrations.

Metabolic studies preliminary results:

To date, mass balance studies have been initiated for radio-labelled BPA, BPZ, Pergafast 201, BPP, (extra: BPS and BPB). Halogenated analogs of BPA are in final purification steps and will be assayed in the forthcoming months. Preliminary results highlight extensive metabolization of several BPA alternatives in HepaRG,as well as pilot studies initiated with microsomes and S9 liver fractions. Work is also ongoing for the development of HPLC-MS conditions allowing the investigation of major metabolites structures.

[ISS (IT)] has developed a **diode-HPLC- array method** to detect and quantify the BPA alternatives, i.e., 1-BPZ, 2-BPE, 5-BPP, 6-BPAP, BPS-MAE and 7-TCBPA, in *in vitro* assays. A PerkinElmer Series 200 Liquid Chromatograph, equipped with a PerkinElmer diode array LC 235 detector and a Restek Pinnacle C-18 DB reversed-phase column (length = 25 cm, diameter = 4.6 mm) were used. The optimal conditions for all BPA alternatives were: 1 mL/min flow rate, 20 µL injection volume, λ = 225 nm and a mobile phase consisting of a mixture of MeOH:H_2O both acidified with 0.05% of trifluoroacetic acid (TFA).

Table A1. The diode HPLC array method for BPA alternative detection and quantification

Substance	Method Description	Retention time (Rt) (min)	R^2	Limit of Detection (LOD) µM	Limit of Quantification (LOD) µM
BPA					
BPZ	MeOH/H2O 60:40 (v/v) for 1,5 min; 5 min to obtain a 70:30 ratio (v/v) of; then after 8 min, 3 min of linear gradient to return to the initial conditions and further 3 min to equilibrate the HPLC	10.284	0.995	0.16	0.53
BPE	MeOH/H2O 55:45 (v/v) for 15 min	9.489	0.997	0.16	0.53
BPS-MAE	MeOH/H2O 55:45 (v/v) for 15 min	10.973	0.992	0.35	1.17
Pergafast 201					
BPP	MeOH/H2O 75:25 (v/v) for 2,5 min; 3 min to obtain an 85:15 ratio (v/v) of mixture; then after 3 min, to return to the initial conditions and lastly 3 min to equilibrate the HPLC	10.284	0.997	0.14	0.45
BPAP	MeOH/H2O 65:35 (v/v) for 15 min	10.433	0.993	0.32	1
TCBPA	MeOH/H2O 75:25 (v/v) for 15 min	9.933	0.993	0.24	0.8
TBBPA					

BPA alternatives were determined referring to a calibration curve prepared with at least 7 known amounts of the analytical standards (0.5–50 µM). The method will be applied in the i metabolism and biokinetic studies.

A SOP for sample preparation and biokinetic analysis was prepared and uploaded in the Sharepoint for the harmonized application of this method across additional cell systems/medium used by partners.

Further result details

(ISS (IT)) - Isoform specific metabolism study has been carried out on BPZ. Different BPZ concentrations (5-10-20-40 µM) and incubation times (0-10-20-30-60 min), using both buffers (50 mM sodium phosphate and 50 mM Tris-HCl), were tested. No plastic adsorption on PPP test tubes (Eppendorf used to centrifuge the samples before the analysis) has been evidenced. No reliable data were obtained on BPZ metabolism due to stability problem of the test item in the incubation buffer and its low solubility in MeOH (this solvent can be used for incubation with recombinant enzymes and Human Liver Microsomes, while DMSO can interfere with the enzymatic activity). BPZ has a reduced solubility in MeOH (tested 10 mM), whereas in DMSO at least 100mM. Calculated solubility in water reported in the literature was 14-60 µM (https://go.drugbank.com/drugs/DB07485ùù; Chemspider); in 50 mM sodium phosphate and Tris-HCl (both at pH=7,4) a lower solubility was expected. The solubility seemed to be further reduced at 37 °C. In addition, the concentration of 40 µM at 1h incubation (max time tested) was decreased by 20% in the absence of any enzymes, indicating chemical instability under the incubation conditions. The loss of BPZ was proportional to incubation time but not reproducible among different replicates, with an extremely high variability mainly related to solubility issues. For this reason, the decrease of BPZ has been considered an inappropriate metric to quantify BPZ metabolism or clearance metabolite formation. To increase solubility of BPZ, BSA was added to the incubation medium: the solubility seemed to increase, but the variability remained, and it

was not clear if BSA could also affect BPZ bioavailability. No new peak, attributable to glucuronide formation, was detected when recombinant UGTs and human liver microsomes were used.

Next step will be to use BPE as the test item, which shows higher solubility in water matrix, to obtain metabolic kinetic data.

Endocrine effects

Steroidogenesis assay

The steroidogenesis assay was performed according to the OECD TG 456 (https://www.oecd.org/en/publications/test-no-456-h295r-steroidogenesis-assay_9789264122642-en.html), using the human adreno-carcinoma cell line (NCI-H295R cells from ATCC). Cells were maintained in DMEM/F12 medium supplemented with 1% ITS-Premix, 2.5% Nu-Serum, 1% Pen-Strep, 15 mM HEPES at 37°C, 5% CO_2 in a humified atmosphere. At the end of 2023, the Corning company stopped producing the Nu-Serum causing a delay in the experimentation. We then substituted it with Nu-Serum IV, containing fetal bovine serum rather than newborn calf serum. To verify that the reproducibility of the assay was not affected, we compared the results of the quality control plates obtained using the two serums and including: non-treated and vehicle treated (DMSO 0,1%) cells, cells treated with the positive control forskolin (at 1 and 10 µM) and the negative control prochloraz (at 0.1 and 1 µM). The results were identical, assuring the robustness of the assay.

All the chemicals (BPA, BPE, BPZ, BPP, BPAP, BPS-MAE, TCBPA, TBBPA, as well as forskolin and prochloraz) were dissolved in 2 ml DMSO in glass amber vials to obtain 100 mM stock concentrations, with no solubility problems. Seven 10-fold diluted concentrations were also prepared in DMSO in glass amber vials; all the solutions are stored at -20°C.

To perform the assay, cells were used from passages 5 to 10; 150,000 cells/cm^2 were plated in 24-wells plates in 1 ml complete medium. The next day, 1 µl DMSO (as vehicle control) or 1 µl 1000x concentrated stock solutions were added in the wells in triplicate. After 48 h incubation at 37°C, conditioned medium of each well was collected and stored at -80°C until analysis, whereas cell monolayers were assessed for vitality by performing the MTS assay. Concentrations decreasing vitality more than 20% were excluded from the hormone assessment; whenever possible, additional intermediate concentrations were added for a more precise assessment. For each compound, three independent experiments at different cell passages were performed. Cytotoxicity IC50 values were calculated with the drc package v3.0 in R 4.3.3.

Commercial ELISA kits were used to assess E2 and testosterone levels. Samples were diluted 1:50 or left undiluted for E2 and testosterone assessment, respectively. The hormone interference test was performed according to the OECD TG 456 protocol; at the maximum concentration tested, the compounds interfered with hormone assessment by less than 20%, as required.

Thyroid Hormone (T3) transport via the MCT8 Transporter Assay

The T3 transport assays was conducted based on the OECD assay (under validation). MCT8 overexpressing cell line (MCDK1-MCT8) and control MDCK1 cells lines were used and maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), 1% Pen-Strep at 37 ºC, 5% CO_2 in a humidified atmosphere.

All the chemicals (BPA, BPZ, BPE, BPS-MAE, PERGAFAST 201, BPP, BPAP, TCBPA, TBBPA, BPH, BPS, BAGDE, Trans-CBDO, and positive control Silychristin) were dissolved in DMSO at stock concentration of 100 mM and stored at –20 ºC until use.

To perform the assay, 50,000 cells/well were seeded in 96-wells plates in 0.25 ml complete medium. After 24 hours, the medium was replaced with an uptake buffer containing 5 µM of T3 and various concentration of test substances (ranging from 0.01 – 1000 µM). The cells were incubated 30 min at 37°C, then washed twice with PBS. Iodine-containing molecules were digested using ammonium persulfate to release the iodide, which was quantified using the colorimetric Sandell-Kolthoff reaction. Each substance was tested in triplicate and repeated in at least three independent experiments. IC_{50} values were calculated with a curve-fitting algorithm in GraphPad Prism. All substances were tested simultaneously using MDCK1-MCT8 and control MDCK1 cells, alongside the potent inhibitor Silychristin as a positive control for the assay.

Iodide (I-) transport via the NIS symporter Assay

The I- transport assay was conducted based on the OECD assay (under validation). Human NIS (Sodium/Iodide symorter) overexpressing cell line (MCDK1-hNIS) and control MDCK1 cells lines were used. Both cell lines were maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), 1% Pen-Strep at 37 ºC, 5% CO_2 in a humidified atmosphere.

All the chemicals (BPA, BPZ, BPE, BPS-MAE, PERGAFAST 201, BPP, BPAP, TCBPA, TBBPA, BPH, BPS, BAGDE, Trans-CBDO, and positive control perchlorate (ClO4-)) were dissolved in DMSO at stock concentration of 100 mM and stored at –20 ºC until use.

To perform the assay, 150,000 cells/cm^2 were seeded in 96-wells plates in 0.25 ml complete medium. After 24 hours, the medium was replaced with an uptake buffer containing 100 µM of I- and various concentration of test substances (ranging from 0.01 – 1000 µM). The cells were incubated 1 hour at 37 ºC. Then, washed twice with PBS. Iodine was quantified using the colorimetric Sandell-Kolthoff reaction. Each substance was tested in triplicate and repeated in at least three independent experiments. IC_{50} values were calculated with a curve-fitting algorithm in GraphPad Prism. All substances were tested in parallel using MDCK1-hNIS and control MDCK1 cells, alongside the potent inhibitor ClO4- as a positive control for the assay.

Immunotoxicity

To determine cytotoxicity and select the concentrations to be tested, PBMC (1x10^6 cells/mL) were cultured in a complete medium consisting of RPMI 1640 without phenol red, 2 mM L-glutamine, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 10 µg/mL gentamycin, and 50 µM 2-mercaptoethanol, supplemented with 5% heated-inactivated delipidated FBS. The cells were treated in a 96-well plate with increasing concentrations of the selected BPA analogues and DMSO as a vehicle control and then incubated at 37°C in 5% CO_2 for 24 hours. For the determination of the leukotoxicity, the CyQUANT™ LDH Cytotoxicity Assay Kit was used (InvitrogenTM Corporation, Massachusetts, US) and the manufacturers procedures followed. Based on the results obtained, the CV80 values were selected, and the concentrations to be used in subsequent tests were determined. In table 5b in the results, the CV80 are reported [UMIL (IT)].

To investigate the effect on T-independent antibody production, PBMC (1.26x10^6 cells/mL) were cultured in a complete medium consisting of RPMI 1640 without phenol red, 2 mM L-glutamine, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 10 µg/mL gentamycin, 50 µM 2-mercaptoethanol, and supplemented with 5% heated-inactivated delipidated FBS. The cells were treated in a 48-well plate with increasing concentration selected base on the CV80 (reported in the results) and DMSO as a vehicle control. They were then incubated at 37°C in 5% CO_2 for 24 hours. Subsequently, PBMCs were stimulated, or not, with 1 µg/mL of ODN2006 and 100 IU/mL of rhIL-2 for an additional 5 days. To determine the release of total IgG and IgM, after a total of 6 days, PBMCs were centrifuged for 5 minutes at 25°C at 380 *g*-. Supernatants were collected and stored at -20°C until measurement. The release of immunoglobulins IgG and IgM was assessed using an ELISA technique. The optical densities were measured using the Molecular Devices SpectraMax ABS, and data were collected and analyzed using integrated software. The results were expressed as fold-change of chemical-treated cells versus vehicle-treated cells [UMIL (IT)].

To assess NK-cell lytic activity, PBMCs (5 × 10^6 cells/mL) were exposed to BPA and its analogues or DMSO for 24 hours. K562 cells, stained with CellTrace™ CFSE, were used as target cells. Briefly, 500 µL of K562 cells at a concentration of 10^6 cells/mL were centrifuged, CellTrace™ CFSE was added to the cell pellet (diluted 1:1000), and the mixture was incubated for 15 minutes at 37°C, protected from light. After incubation, the reaction was halted by adding culture medium containing 5% heat-inactivated dialyzed fetal bovine serum. The CellTrace™ CFSE-stained K562 cells were then adjusted to a concentration of 10^5 cells/mL and co-cultured with BPA analogues/DMSO-exposed PBMCs. Three different effectors (PBMC) to target (K562) cell ratios were used: 50:1, 25:1, and 12.5:1, while maintaining a constant concentration of K562 cells. The cells are then co-cultured for 4 h at 37°C in a 5% CO_2 incubator. To evaluate the lytic activity of NK cells, the plate contents were transferred to flow cytometry tubes, and PI (5 nM) was added to each tube. The percentage of PI-positive cells among CFSE-stained

K562 cells was then measured using a Novocyte 3000 flow cytometer. In total, 1,000 CFSE-positive cells were analyzed for PI positivity, indicating the presence of dead K562 cells [UMIL (IT)].

In silico screening [UL FFA]

In silico screening of BPA and its 25 substitutes using Endocrine Disruptome (http://endocrinedisruptome.ki.si/, https://pubmed.ncbi.nlm.nih.gov/24628082/) was performed in order to evaluate their effect on nuclear receptors. Since endocrine disruption might be a contributing factor to immunotoxicity, the compounds being predicted to react with several nuclear receptors were expected to exhibit higher immunomodulatory properties. The software is able to estimate the binding affinity of small molecules to 12 nuclear receptors, i.e., androgen receptor (AR), estrogen receptors α (ERα) and β (ERβ), glucocorticoid receptor (GR), liver X receptors α (LXRα) and β (LXRβ), mineralocorticoid receptor (MR), peroxisome proliferator-activated receptors α (PPARα), β (PPARβ) and γ (PPARγ), progesterone receptor (PR), retinoid X receptor α (RXRα), thyroid receptors α (TRα) and β (TRβ). For each receptor (including agonistic and antagonistic mode of action for AR, GR and ERs), compounds were ranked from 1 to 26, where first place represents the highest binding potential. Then, the comprehensive ranking was performed where the sum of all the ranks for each compound was calculated. The compounds with the lower sum present the higher potential for endocrine disruption.

In vitro assays

As a model of human macrophages, THP-1 cells were differentiated into macrophages with exposure to 80 nM phorbol-myristate-acetate (PMA) for 72 h and used as such for the experiments. Jurkat T cells were used as a model for human T lymphocytes and lymphoblastoid cell lines (LCLs) as a model for B lymphocytes.

For the assessment of the BPA alternatives on metabolic activities, the cells were exposed to the increasing concentrations of substitutes or vehicle control for 24 h. Then, their metabolic activity was evaluated by the resazurin method. The IC50 values were calculated using GraphPad Prism.

For the assessment of the BPA substitutes on the cytokine release, the cells were pretreated with the substitutes for 2 hours and then appropriately stimulated for 24 h. The released cytokines were measured in the cell culture supernatants using multiplex bead arrays (macrophages, LCLs) of ELISA (Jurkat). Specifically, THP-1 derived macrophages were exposed to 10 nM, 100 nM or 10 μM of the compounds and their effects on IL-1β, IL-6, IL-8 and TNFα were assessed. Jurkat T cells were exposed to the increasing μM concentrations of compounds and the IC50 values of IL-2 inhibitions were determined. LCLs were exposed to 100 nM or 10 μM compounds and their effects on IL-2, IL-6, IL-10 and TNFα were assessed. The cytokines released from THP-1 derived macrophages and LCLs were compared to the vehicle controls and statistical analysis was performed by Dunnett's multiple comparisons.

Immune Cells Calcium Homeostasis [ISS(IT)]

Jurkat T cells were employed as a model for human T lymphocytes. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 IU/mL penicillin and 0.1 mg/mL streptomycin) at 37°C in a humidified atmosphere with 5% CO_2. The experimental procedure for $[Ca^{2+}]_i$ measurement was adapted from established protocols, and specific SOPs were developed. On the day of the experiment, cells were loaded with the fluorescent calcium-sensitive probe Fura-2, suspended in a HEPES-buffered saline working solution, and kept on ice in the dark until use (for a maximum of 4 hours). An aliquot of approximately 1.5×10^6 cells was then transferred into a spectrofluorometer cuvette containing 1.5 mL of working solution at 37°C under gentle agitation. After signal stabilization (~1 minute), time-based fluorescence acquisition was initiated. To evaluate the acute effects of the analouges on resting $[Ca^{2+}]_i$, cells were challenged with the selected analouge or with the vehicle alone (0.1% DMSO), and fluorescence signals were recorded for the following five minutes. To evaluate the acute effects of the analouges on calcium signaling, cells were exposed to the analouge for two minutes and then stimulated with OKT3, an anti-CD3 monoclonal antibody that induces a calcium response by activating the T cell receptors. At the end of each experiment, the signal was calibrated to convert arbitrary fluorescence units into calcium concentration units ($[Ca^{2+}]_i$). Finally, cell responses were quantified in terms of area under the curve. Long-term effects were assessed by incubating cells with the analouges (or 0.1% DMSO) in complete cell culture medium without phenol red for 24 hours at 37°C, 5% CO_2, in a humidified atmosphere. At the end of the incubation, basal $[Ca^{2+}]_i$ and OKT3-induced calcium responses were measured as described above.

Control experiments without cells were always performed to assess potential interference from autofluorescence and/or scattering phenomena caused by the dispersion of the substances into the buffer. Cell viability under the different assay conditions was assessed using trypan blue.

Developmental Neurotoxicology

Hippocampal Neurons in vitro screening by [CNRS (FR)]

The generation of hippocampal neurons from hiPSCs was set up by adapting previously reported protocols. We performed three differentiations of two distinct hiPSCs clones into hippocampal neural progenitors (hNPCs), which then undergo neuronal maturation. We performed RT-qPCR analyses to confirm successful hippocampal differentiation. We showed that octamer-binding transcription factor 4- (OCT-4) expressing hiPSCs differentiated with high efficiency into hNPCs expressing NPC marker Nestin and hippocampal progenitor markers prospero homeobox 1 (PROX1) and zinc finger and BTB domain containing 20 (ZBT20). Furthermore, the neuronal marker microtubule associated protein 2 (MAP2), and hippocampal markers ELAV like RNA binding protein 2 (ELAVL2) and glutamate ionotropic receptor kainate 4 (GRIK4) were expressed in mature hippocampal neurons at day 43.

These hNPCs were treated chronically with increasing doses (0.001 µM, 0.01 µM, 0.1 µM, 1 µM, 10 µM, 100 µM) of BPA, BPAP, BPE and BPZ over a 14-day period. Cell viability tests were conducted. All the analyzed bisphenols were associated with a concentration-dependent decrease in cell viability, with a significant decrease observed at doses of 100 µM for BPA, BPAP and BPZ and from 10 µM for BPE. On the basis of the results of these tests, non-toxic doses (0.001 µM, 0.01 µM, 0.1 µM, 1 µM) were chosen to treat the NPC with bisphenols for 14 days.

Immunocytochemistry for β-III tubulin has been performed, and analysis of neuronal morphology (number of neurites per neuron, length and number of branches) is currently underway. In addition, protein levels of presynaptic (synaptophysin, synapsin 1) and postsynaptic (PSD95, gephyrin, glutamate receptors) markers will be quantified by Western blot to measure the impact of treatments on synaptic plasticity. RNA and DNA have been extracted at the end of the treatment for analyses of gene expression and DNA methylation.

In parallel with these analyses, cell viability tests for BPA, BPP, BPS-MAE and TCBPA will start soon, before conducting the same analyses as for BPA, BPAP, BPE and BPZ.

SOPs for these analyses are under progress.

Neurosphere Assay (In Vitro Test Battery for DNT) by [IUF (DE))

The analysis was performed by making use of the Neurosphere assay (Koch et al. 2022), a medium throughput and high content in vitro assay for developmental neurotoxicity (DNT), and an integral part of the developmental neurotoxicity in-vitro testing battery (DNT-IVB; Blum et al. 2023; OECD377 2023). It utilizes primary fetal human neural progenitor cells (hNPCs) cultured as neurospheres, 3-dimensional cell aggregates with the potential of differentiating into various brain effector cells (i.e. neurons, astrocytes, and oligodendrocytes). The assay models seven critical early human neurodevelopmental key events associated with DNT: hNPC proliferation (NPC1); radial glia, neuronal and oligodendrocytes migration (NPC2a/b/c respectively); neuronal differentiation (NPC3); neurite outgrowth (NPC4) and oligodendrocyte differentiation (NPC5).

In the NPC1 assay, evaluating the hNPC proliferation, neurospheres were cultured in suspension, over 3 days. The cells were exposed to the tested chemicals from day 0, at 7 different concentrations (20, 6.6, 2.2, 0.74, 0.24, 0.082, 0.027 µM) using four technical replicates each. On day 3, hNPC proliferation was assessed by two methods: increase of sphere area (NPC1a) and 5′-bromo-2′-deoxyuridine (BrdU) incorporation in the DNA (NPC1b). Cytotoxicity and mitochondrial activity were assessed on day 3, in order to discern between unspecific hits (i.e. caused by cytotoxicity) and specific hits (i.e. caused by a substance-related mode of action that differs from cytotoxicity or reduced viability). Unless stated otherwise in table xx, at least three biological replicates were conducted for each substance.

The NPC2-5 assay is a multiplexed assay used to evaluate key neurodevelopmental events related to DNT. Neurospheres were cultured on an extracellular matrix and allowed to differentiate for five days. Chemical exposure started at day 0 and continued throughout the assay duration of five days, 7 concentrations were tested (20, 6.6, 2.2, 0.74, 0.24, 0.082, 0.027 µM) with four technical replicates per condition. At day 3, radial glia migration

(NPCa) and cytotoxicity were assessed, and at the same time, cell culture medium with chemicals was renewed. On day 5 cytotoxicity and mitochondrial activity assessment was performed, after which, the cells were fixated, and immunohistochemical staining was performed to identify differentiated neurons and oligodendrocytes. To calculate NPC2b/c, NPC3, NPC4, and NPC5 endpoints, stained neurons and oligodendrocytes were identified by Omnisphero, an in-house software, in combination with two convolutional neural networks (CNN), based on the Keras architecture implemented in Python 3. Unless stated otherwise in table xx, at least three biological replicates were conducted for each substance.

Carcinogenicity

Cell Transformation Assay by [INSA (PT), NILU (NO), INRS (FR), FIOH (FI)]

This assay was conducted following the OECD Guidance Document on the In Vitro Bhas 42 cells Transformation Assay, 2016.

Bhas 42 cells were thawed and grown in DMEM supplemented with 10% FBS until approximately 70% confluence and then were trypsinized and grown in DMEM F-12 supplemented with 10% FBs and 1% Penicilin/Streptomycin (100,000 units/L Penicillin G sodium and 10 mg/mL streptomycin sulfate) until reaching 70% confluence. At day 0, cells were seeded in 6-well plates at 4000 cell/well for the initiation test and at 14,000 cell/well for the promotion test. Six wells were prepared per treatment condition. For the concurrent cell growth assay, 96-well plates were also seeded with 200 cells/well for initiation and with 400 cells/well for promotion. Eight wells were prepared per treatment condition.

For the initiation test, 24h after seeding (day 1), treatment medium containing bisphenols (máx. DMSO 0.25%) were added to the cultures. 3-Methylcholanthrene (MCA) at 1 µg/mL was used as a positive control for the initiation test. The treatment was continued for 72h. At day 4, all treatment media were removed, and the cells were post-cultivated in fresh medium until day 7 (concurrent cell growth) or until day 21, receiving medium exchanges on day 7, 11 and 14.

For the promotion test, at day 4, culture medium was replaced with treatment medium containing bisphenols (máx. DMSO 0.25%). 12-O-tetradecanoylphorbol 13-acetate (TPA) at 0.05 µg/mL was used as a positive control for the promotion test. The treatment was continued for 72h and removed at day 7 (concurrent cell growth) or renewed at day 7 and 11. At day 14, all treatment media were removed, and the cells were post-cultivated in fresh medium until day 21.

DMSO at 0.1 and 0.25%, as well as 5% H_2O and caffeine at 100 µg/mL, worked as negative controls for both initiation and promotion.

Concurrent cell growth assay ended at day 7 for both initiation or promotion tests, and viability tests were conducted on these cultures to ascertain cells viability in the concentrations tested. MTT assay, Alamar Blue or WST-1 are some of the viability assays that were performed. Results were expressed as % of viability compared to the vehicle control.

At day 21, for initiation and promotion, culture medium was removed and cells were fixed with absolute ethanol, and stained with a 5% Giemsa solution for 30 min. and then rinsed with tap water. After drying overnight, plates were ready for foci scoring. The transformed foci were evaluated according to the criteria described by Sakai et al. (2011), Sasaki et al. (2012) and OECD Guidance Document (2016). The results in the Bhas 42 cell transformation assays were positive when inducing statistically significant increases in the number of transformed foci compared to the respective vehicle control.

A.3 Methods: BPA alternatives environment

Aquatic invertebrates

Activities (i) & (ii) tests	**SLU** (SE)	**IISPV** (ES)	**SDU** (DK)	**INERIS** (FR)	**IEP-NRI** (PL)	**BPI** (GR)	**UG-PL** (PL)	**UAVR** (PT)	**SU** (SE)	**Cefas** (UK)
Acute toxicity test on *Daphnia magna* (OECD TG 202)	n/r	BPS, BPS-MAE, BPAP, BPE (scheduled but not finalised yet)	n/r	Not initially scheduled but it is stated in the report that BPA, BPF, BPS and BPB have been tested	No data presented for single compounds (mixtures)	BPB, BPF, BPS, BPAF	No data presented for single compounds (mixtures)	No data presented for single compounds (mixtures)	BPA, BPZ, BPE, BPF, BPS, BPAF	n/r
Daphnia magna reproduction test (OECD TG 211, level 4 of OECD CF)	n/r	n/r	n/r	n/r	No data presented for single compounds	n/r	No data presented for single compounds (mixtures)	No data presented for single compounds (mixtures)	BPA, BPAF, BPS, BPF	n/r
Short-term Juvenile Hormone Activity Screening Assay using *Daphnia magna* (JHASA, OECD 253)	n/r	n/r	n/r	BPA, BPF, BPS, BPB	n/r	n/r	n/r	No data presented for single compounds	n/r	n/r
Lymnaea stagnalis Reproduction Test (OECD TG 243)	BPA, BPZ, BPS-MAE	n/r	BPE BPAP	n/r	n/r	n/r	n/r	n/r	n/r	n/r
Regeneration assay using the freshwater planarian *Girardia tigrina*	n/r	n/r	n/r	n/r	n/r	n/r	n/r	BPA, BPAF, BPS-MAE, BPZ	n/r	n/r
10-day acute sediment toxicity bioassay with the marine polychaete Arenicola marina	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	BPA, BPAF, BPAP, BPZ
42-day chronic toxicity test using the freshwater amphipod Hyalella azteca, spiking sediment	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	BPAF

n/r: not relevant

Zebrafish

Activities (i) & (ii) tests	**INERIS** (FR)	**NIB** (SI)	**UU** (SE)	**BPI** (GR)	**UAVR** (PT)
Fish Embryo Acute Toxicity Test (OECD TG 236)	BPA, BPB, BPE, BPC, BPC-Cl, BPF, BPS, BPS-MAE, BPS-MPE, BPZ, TCBPA, BPAF, BPAP, 4-4'ODP	BPA, BPAF, BPPH, BPAP	n/r	BPS, BPB, BPF	BPE, BPZ, BPS-MAE
Fish short-term reproduction assay (OECD TG 229, level 3 of OECD CF), or 21-day fish assay: a short-term screening for estrogenic and androgenic activity and aromatase inhibition (OECD TG 230, level 3 of OECD CF)	It was initially planned to assess the endocrine and reproductive effect of one bisphenol substitute in the OECD TG N°229 but it was not possible to perform this experiment due to "human factors" beyond partner's control."	n/r	n/r	n/r	n/r
EASZY assay: Detection of substances acting through Estrogen Receptors Using Transgenic cyp19a1b GFP Zebrafish Embryos (OECD TG 250, level 3 of OECD CF)	BPA, BPB, BPE, BPC, BPC-Cl, BPF, BPS, BPS-MAE, BPS-MPE, BPZ, TCBPA, BPAF, BPAP, 4-4'ODP	n/r	n/r	n/r	n/r

n/r: not relevant

Alga / aquatic plants

Activities (i) & (ii) tests	**IISPV** (ES)	**IEP-NRI** (PL)	**BPI** (GR)	**UG-PL** (PL)	**SU** (SE)
Algae growth inhibition test on *Raphidocelis subcapitata* (formerly known as *Selenastrum capricornutum*) (OECD TG 201)	No data presented yet for single compounds	No data presented yet for single compounds	BPB, BPS, BPF	n/r	BPAF, BPS, BPF, BPA
Lemna sp. Growth Inhibition Test (OECD TG 221)	n/r	BPZ, BPP, BPE	n/r	No data presented yet for single compounds (mixtures) Initially scheduled to test: BPS, BPE, BPA, BPF	n/r

n/r: not relevant

Soil organisms

Activities (i) & (ii) tests	**IISPV** (ES)	**INERIS** (FR)	**BPI** (GR)
Effects on soil micoorganisms: Carbon transformation test (OECD TG 217 and ISO 11274)	BPS, BPS-MAE, BPAP, BPE, BPZ, Pergafast 201, BPP, BPPH (no endpoints are available yet since the statistics were not finalized)	n/r	n/r
Effects on soil microorganisms: Nitrogen transformation test (OECD TG 216)	BPS, BPS-MAE, BPAP, BPE, BPZ, Pergafast 201, BPP, BPPH (on going)	n/r	n/r
Earthworm reproduction test (OECD 222)	n/r	n/r	BPE, BPZ (on going experiments)

n/r: not relevant

Amphibians

Activities (i) & (ii) tests	**UAVR** (PT)
In vitro assays with A6 epithelial-like cells (ECACC 89072613) of Xenopus laevis	BPA, BPAF, BPZ, BPE, BPS-MAE
Behaviour toxicity assay with larvae of Xenopus laevis and zebrafish eleutheroembryos	BPA, BPAF, BPZ, BPS-MAE (results still being analysing for zebrafish)
Frog embryo teratogenicity assay (ASTM E1439-12)	BPA, BPAF, BPZ, BPE, BPS-MAE
Xenopus Eleutheroembryo Thyroid Assay XETA (OECD TG 248, level 3 of OECD CF)	No data has been presented yet

n/r: not relevant

In vitro / high throughput assays

Activities (i) & (ii) tests	**IISPV** (ES)	**IEP-NRI** (PL)	**BPI** (GR)	**UAVR** (PT)	**Eawag** (CH)	**UFZ** (DE)
Fish Cell Line Acute Toxicity - The RTgill-W1 cell line assay (OECD TG249)	n/r	n/r	n/r	n/r	BPA, BPE, BPF, BPS, BPS-MPE, BPS-MAE, BPB, BPAP, BPAF, BPZ,	n/r

Activities (i) & (ii) tests	**IISPV** (ES)	**IEP-NRI** (PL)	**BPI** (GR)	**UAVR** (PT)	**Eawag** (CH)	**UFZ** (DE)
					BPP, bisOPP-A, TBBPA, TCBPA*	
Microtox® Acute Toxicity Test	n/r	BPAF, BPF, BPS-MAE, BPS-MPE, BPPH	BPAF, BPF, BPS-MAE, BPS-MPE, BPAP, BPP, BPPH, BPE, BPZ, Pergafast	n/r	n/r	n/r
YES assay, in vitro screen with Saccharomyces cerevisiae	n/r	n/r	n/r	BPA, BPE, BPAF, BPZ, BPAP, BPG, BPS, BPS-4-allyl ether	n/r	n/r
Reporter gene assays and mammalian cell models of neurotoxicity for high-throughput screening	n/r	n/r	n/r	n/r	n/r	n/r

n/r: not relevant