Unveiling plastic food packaging chemicals' mixtures: a combined effect-directed strategy to assess environmental exposure and health impacts

Writing assignment Research Proposal

Name student:	Adele Selma Ferrario (9145419)
Affiliation:	Utrecht University, IRAS
Name first examiner:	Ingeborg Kooter
Affiliation:	Circular Economy and Environment Research Group, TNO
Name second examiner:	Gerard Hoek
Affiliation (university/institute + department):	Institute for Risk Assessment Sciences, Utrecht University

Milou Dingemans from Water Research Institute (KWR) and guest researcher at IRAS (UU) and Misha Vrolijk, Assistant Professor at the Faculty of Health, Medicine and Life Sciences, Pharmacology and Toxicology at Maastricht University, were also consulted during the research proposal writing.

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Abbreviations

2,4-DTBP - 2,4-di-tert-butylphenol

AOP - adverse outcome pathways

CMR - carcinogenic, mutagenic, or toxic for reproduction

CTS - Chemical Transformation Simulator

BEQ - bioanalytical equivalent concentrations

BPA - bisphenol A (BPA)

DBP - dibutyl phtalate

DEHA - di(2-ethylhexyl) adipate

DEHP - di(2-ethyl hexyl) phthalate

DEP - dimethyl phthalate

EBM - effect-based monitoring

EFSA - European Food and Safety Authority

ECHA - European Chemical Agency

EDC - endocrine disrupting chemical

EQS - Environmental Quality Standard

FCM - food contact materials

GC - gas chromatography

HPLC - high-performance liquid chromatography

HR-MS - high-resolution mass spectrometry

IAS - intentionally added substance

MoA - mode of action

NAMs - new approach methodologies

NIAS - non-intentionally added substance

NTA - non-targeted analysis

PAE - phthalate esters

PBT - persistent, bioaccumulative and toxic

PBK - physiologically based kinetic

PE - polyethylene

PET - polyethylene terephthalate

PMT - persistent, mobile, and toxic

PoD - point of departure

QIVIVE - *in vitro*-to-*in vivo* extrapolation

REACH - Registration, Evaluation, Authorisation and Restriction of Chemicals

SML - specific migration limit

SVHC - substance of very high cncern

TA - targeted analysis

TDI - tolerable daily intake

TOTS - Trade-off Tool for Sampling

TP - transformation product

UV - ultraviolet

vPvM - very persistent, very mobile

vPvB - very persistent and very bioaccumulative

WHO - World Health Organization

Abstract

Plastic packaging is a dominant sector within the plastic industry, primarily finding applications in food packaging. Chemical substances are intentionally incorporated into plastic food packaging to impart specific product characteristics or streamline production in compliance with EU No 10/2011. However, unintentional substances can also be present as contaminants or tramsformtion products. These substances may leach from the packaging, particularly during reuse, recycling, exposure to elevated temperatures an ultraviolet radiation (UV) or microwaving, leading to contamination of the stored food and beverages and the surrounding environment, including air, water, soil, and sediments. Notably, some authorised chemicals that can migrate from plastic food packaging, such as bisphenol A and phthalate esters, are already recognised for their high toxicological risk. The core issue lies in the limited comprehension of these mixtures' chemical composition, exposure levels, and toxicological properties, which raises substantial concerns about potential human health risks. Our research investigates these aspects by applying a combined effect-directed methodology. Migration experiments under varying experimental conditions and environmental sampling at critical locations will be conducted. One of our key objectives is to unveil the essential factors influencing the migration of chemicals from plastic food packaging and to illuminate the types of packaging and conditions most susceptible to this phenomenon. *In vitro* assays will be implied to account for ingestion and inhalation exposure routes and assess hazard endpoints such as genotoxicity and endocrine disruption. This approach will enable us to prioritise mixtures of concern for subsequent chemical analysis. To uncover the compositions of these mixtures, these will be attentively identified by employing non-target screening alongside computational predictions for identifying unknown signals. By combining chemical analysis, *in vitro* approach, chemical analysis and predictive tools, this project aims to construct a comprehensive framework for effect-directed evaluation of plastic food packaging-associated chemicals' mixtures.

Layman’s summary

Plastic food packaging has significantly enhanced our lives by providing convenience and extending the shelf life of our favourite food and beverages. However, these products are not composed solely of plastic. Manufacturers intentionally introduce a range of chemicals to confer specific properties to plastic or facilitate the production process. Additionally, unintended chemicals can be present, including contaminants or newly formed substances resulting from chemical reactions within these added compounds. Some of these chemicals can migrate from the packaging over time, especially after reuse or recycling and at high temperatures, solar exposure, and microwaves. The migration of these chemicals could contaminate the food and beverages they come into contact with and the surrounding environment when they become waste. Given the widespread prevalence of these chemicals, there is a likelihood that we unknowingly ingest or inhale them. Despite this,

our understanding of these chemicals and their potential health risks remains limited. While some chemicals, such as bisphenol A and phthalates, are recognised as problematic for human health, they continue to be employed because finding suitable alternatives is not straightforward.

Our research aims to identify mixtures of chemicals capable of migrating from the packaging into our food and beverages and ending up in the environment after use. We will assess whether these chemicals are absorbed by our bodies via ingestion or inhalation using specialised cells that mimic our intestines and lungs, and their effects on cells to select mixtures raise toxicological concerns. If a chemical mixture has the potential to be absorbed by ingestion or inhalation, and it is dangerous for cells, it could pose a risk to human health.

Once chemical mixtures of concern are identified, we will conduct a chemical analysis to determine the constituents of those mixtures and compile a list of chemicals potentially threatening human health. Computational predictions will be employed to identify newly formed chemicals that might not be present in chemical databases and to identify signals of the chemical analyses that are not otherwise interpreted.

A methodology for evaluating other chemicals of concern in the future will be proposed base on the applied ests on cells combined with analytical methods and their results. Such a framework is crucial for the research, policy, and industry sectors, as it necessitates additional methodologies to assess the health risks associated with emerging chemicals of concern, thereby safeguarding both human health and the well-being of our planet.

Keywords

Plastic food packaging, chemical mixtures' migration, bioassays, non-targeted analysis, effect-directed methods

1. Research topic

1.1 Background

Plastic has found manifold applications in everyday life, with plastic packaging being the dominant market (Groh et al., 2019). Plastic packaging alone represents 40% of European plastic production demand and is responsible for approximately half of the total global plastic waste ever generated (Geyer et al., 2017). Among different applications, most plastic packaging is used for food and beverages (Groh et al., 2019), and most food packaging is destined to end its life within a year of its production (Geyer et al., 2017).

Plastics are complex mixtures of chemicals (Muncke, 2021). Chemicals are authorised to be intentionally incorporated into the primary polymer structure of plastic packaging to obtain specific characteristics, i.e. flexibility, low flammability, and resistance to heat-related deterioration (Groh et al., 2019). Plasticisers, fillers, and flame retardants account for about three-quarters of all additives (Geyer et al., 2017), followed by colourants, stabilisers, and lubricants (Groh et al., 2019). Moreover, production aids can be used during manufacturing, embedding polymerisation catalysts, solvents, and lubricants (Wiesinger et al., 2021).

In the European Union, the *EU No 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food* defined the Union List of Authorised Substances, the positive list of 1'072 chemicals that can be used as intentionally added substances (IAS) authorised for use for all types of plastic food materials and their specific migration limits (SMLs), included because the total absence of migration is unrealistic (Meng et al., 2023).

The legislation also declares that other non-intentionally added substances (NIAS) '*may be present in the material or article but not included in the Union list*' (Article 18, EC 10/2011), and they '*have not necessarily been risk assessed as they had not been subject to an authorisation procedure*' (Article 42, EC 10/2011). NIAS can be impurities of starting substances, contaminants, by-products and reaction products (Meng et al., 2023), collectively called transformation products (TPs). For instance, plastic additives have been found to degrade and transform into new compounds (Groh et al., 2021; Barrick et al., 2021; Bridson et al., 2021; da Costa et al., 2023; Muncke et al., 2021).

However, sometimes, discriminating between IAS and NIAS is problematic because it is unclear whether it was intentionally added to the product or present as a consequence of contamination or degradation processes (Gerassimidou et al., 2023), and the chemical analysis becomes challenging (Muncke et al., 2020).

Exposure to chemicals associated with plastic food packaging

Chemicals associated with the polymeric structure - both IAS and NIAS - can be released in the surrounding environment (Groh et al., 2019; Geueke et al., 2022; Meng et al., 2023) as lacking covalent bonds with the polymer (da Costa et al., 2023). Indeed, plastic food packaging can release chemicals over the entire life cycle and contaminate the environment, and newly formed NIAS can be formed for biodegradation or physical degradation of plastic materials (Hahladakis et al., 2018; Gunaalan et al., 2020; Bridson et al., 2021). Human exposure might arise from releasing chemicals associated with plastic products, including plastic additives and adsorbed chemicals, but the characterisation exposure levels is still insufficient (Koelmans et al., 2019).

With the migration to food already being demonstrated decades ago, the degree of chemical transfer from food packaging to food depends on 1) the physicochemical characteristics and properties of the FCM, 2) the physicochemical characteristics of the migrant, 3) the type of food, and 4) conditions involved, such as the temperature and storage time (Weber et al., 2023; Meng et al., 2023). Ageing (Can & Yerlikaya, 2019; Luo et al., 2022), high temperatures (Sewwandi et al., 2023), and reusing and recycling are recognised as critical conditions for migration (Ahmadkhaniha & Rastkari, 2017; Geueke et al., 2023).

Due to this migration, chemicals can contaminate food and beverages in contact with, but also the water, air, and soil since, worldwide, 71% of plastic waste ends up in aquatic or terrestrial environments (Geyer et al., 2017). For example, the disposal of plastic in landfills, which is the main destiny of plastic waste, can result in the release of additives and the pollution of water, air, sediments, and soil, primarily due to ultraviolet (UV) radiation and the infiltration of water and its uptake of chemical mixtures (Maceira et a., 2019; Bridson et al., 2021; Wojnowska-Baryła et al., 2022). Moreover, air pollutants can significantly contribute to the deterioration of polymers and, therefore, the release of associated chemicals (Hahladakis et al., 2018).

Among authorised plastic additives, phthalates (PAEs) have undergone a thorough investigation as they are known to be persistent in the environment (Bridson et al., 2021). PAEs have gained priority status as pollutants due to their potential environmental contamination, encompassing food and drinks, soil, sediment and aquatic environment (Zhang et al., 2015). Due to their relatively low molecular weight, PAEs readily migrate from deteriorating plastic products, with high concentrations found in soil agricultural areas (Viljoen et al., 2023).

Di(2-ethyl hexyl) phthalate (DEHP), dibutyl phthalate (DBP) - two PAEs - and bisphenol A (BPA) are plasticisers authorised in the Union lists to increase the flexibility and durability of plastic FCMs. They are the most commonly detected IAS in food and drinks, with high temperatures a driver of migration (Geueke et al., 2022; Sewwandi et al., 2023). However, this predominance might be due to the focused analytical determination of these substances, as critical from a toxicological point of view. However, many more substances might exist (Geueke et al., 2023).

Interestingly, Wiesinger et al. (2021) found that DBP is not authorised for plastic products in the EU, while it is approved for use in FCMs (Wiesinger et al., 2021). DEHP was the most commonly detected PAE in food samples by Can and Yerlikaya (2019), and the transfer of DEHP and DBP from single-use PE plastic tableware into water was discovered to surpass the recommended limits proposed by the World Health Organization (WHO) (Gerassimidou et al., 2023).

Overall, food contact materials (FCMs) represent a significant route of chronic exposure for humans (Muncke et al., 2020), with great concern for plastic products for their widespread use. Notably, two-thirds of the 1'975 chemicals in contact with food reviewed by Geueke et al. (2022) were associated with plastic FCMs. Gerassimidou et al. (2023) identified 377 chemicals migrating only from (non-multilayer) polyethene (PE) FCMs, among which the majority (211) were found to migrate at least at one of the experimental conditions, and 62 were authorised substances, among which the 25% exceeded the prescribed SML (Gerassimidou et al., 2023).

This problem is even more present when plastic is recycled to produce food-grade plastics, often used as a middle layer of multilayer products (Gerassimidou et al., 2023). The reuse and recycling of plastic can result in the permanence of hazardous chemicals in the plastic FCM (Groh et al., 2019; Geueke et al., 2023). For example, recycled polyethene terephthalate (PET) has been widely used in food contact products in the last 20 years, and release from such materials has been proven to increase over time (Geueke et al., 2023). Geressimidou et al. (2023) highlight that further research on the chemical hazards of recycled PE food packaging is needed because chemicals migrate into food and lack fate and toxicity data.

Commission Regulation EU 2022/1616 of 15 September 2022 on recycled plastic materials and articles intended to come into contact with foods regulates the recycling process. The regulation defines the decontamination process, but it is limited to microbiological risks, and chemical contamination is overlooked, especially for NIAS, as they are formed over time and often are unpredicted (Geueke et al., 2023). Decontamination during a specific recycling process, i.e. during PET recycling for FCMs, is applied to a restricted amount of chemicals but still overlooks the presence of NIAS and known chemicals of concern, such as degradation products and residues of the recycling process or food residues (Tsochatzis et al., 2022).

Micro- and nano-plastics (MNPs) formed by plastic degradation can adsorb chemicals associated with plastic food packaging, with lower concentrations being more adsorbed (de Costa et al., 2023), and become vehicles of mixtures of chemicals, which can be released under certain conditions (Bakir et al., 2014; Bridson et al., 2021; Wojnowska-Baryła et al., 2022), facilitating their transport over long distances (Hamilton et al., 2023) and their bioavailability (Groh et al., 2019; Wojnowska-Baryła et al., 2022). A WHO report decreed that plastic additives from MNPs are associated with no safety concerns (Koelmans et al., 2019). However, only additives with known points of departure (PoDs) were included, which are not defined for all the chemicals, i.e. for

endocrine disruption chemicals (EDCs). Moreover, the data were limited, and the quality analysis was not performed (Koelmans et al., 2019).

The toxicological concern of chemicals associated with plastic food packaging

As highlighted by Wiesinger et al. (2021), only a restricted subset of IAS put on the market has undergone comprehensive scrutiny to evaluate their hazard classification. While some chemicals associated with plastic packaging have already been recognised as of high toxicological concern, among which several chemicals are authorised (Muncke, 2021), the majority of chemicals associated with plastic food packaging's health impacts are not yet characterised (Muncke, 2020, 2021; Sewwandi et al., 2023).

Plastic food packaging associated chemicals have been found to persist in the environment and cause ecotoxicity (Barrick et al., 2019; da Costa, 2023) and potentially human-related health effects (Groh et al., 2019; Muncke et al., 2020). Also Groh et al. (2019) have identified 148 chemicals likely associated with plastic packaging, among which are of concern as labelled as carcinogenic, mutagenic, or toxic for reproduction (CMR), persistent, bioaccumulative and toxic (PBT), very persistent and very bioaccumulative (vPvB substances), endocrine disrupting chemical (EDC), associated with environmental hazards (ENV), or human health hazard (HH). Among the 515 genotoxic or carcinogenic reviewed by Groh et al. (2021), 160 came from plastic FCMs (Groh et al., 2021). Here are reported some examples.

- Among authorised IAS, the class of PAEs attracted toxicological attention as recognised endocrine disruptors toxic at low levels, and their presence as plasticisers in food packaging raises safety concerns (Zhang et al., 2015; Gerassimidou et al., 2023). Plasticisers interfere with the thyroid and growth hormones, impede cholesterol transport and steroid production, and lead to reduced fertility due to increased oxidative stress in aquatic organisms (Mathieu-Denoncourt et al., 2015).
- Another critical plasticiser is bisphenol A (BPA), authorised by the Union List with restrictions on infant products (10/2011/EU, Annex I) found in PVC/PC reusable beverage bottles, bottle tops, and epoxy resin coatings for food cans (Lambré et al., 2023). *In vivo* studies and epidemiological studies have demonstrated that BPA causes genotoxicity, metabolic effects, neurotoxicity, cardiotoxicity, developmental, reproductive, and endocrine disruption effects, and potential carcinogenicity, with the most critical the adverse effect on the immune system (Lambré et al., 2023). BPA actual exposure might not protect human safety, as the tolerable daily intake (TDI) has been lowered by 20.000 in comparison to 2015 by the European Food and Safety Authority (EFSA), decreeing health concerns at the present levels of estimated dietary exposures and warranting attention to reduce exposure (Lambré et al., 2023).
- Among authorised monomers, melamine is mainly used for reusable tableware and utensils, also for childrens' products. However, it is also used in the coating of cans and the production of jar lids. Melamine's migration from repeat-use of food contact plastic articles has been known since the 80s, and migration can increase with temperature, microwaves, and UV radiation (Geueke et al., 2023). It is toxic for humans, labelled as persistent, very mobile (vPvM) and might be neurotoxic (Geueke et al., 2023). It is

under assessment as PBT and EDC, and a suspect carcinogen and included in the candidate list of SVHC (ECHA, 2023).

Accounting as NIAS, dimethyl phthalate (DEP) - another PAE - was found in PET bottles (Wiesinger et al., 2021) and BPA in PE once (Gerassimidou et al., 2023). Also, 2,4-di-tert-butylphenol (2,4-DTBP) is a degradation product of commonly used antioxidants in FCMs, and it has been found at high concentrations in human urine samples (Geueke et al., 2023). It is under assessment as EDC (ECHA, 2023). Another example is surfynol, a surfactant not regulated but commonly used in adhesives implied in food packaging multilayers and recognised as toxic in mammals, possibly reducing male fertility by protein inhibition pathway (Nerin et al., 2018).

The European Chemical Agency (ECHA) aims at controlling and replacing Substances of Very High Concern (SVHC) for Authorisation according to Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) and has listed 235 canidates as found to be CMR, PBT, vPvB, or chemicals of equivalent concern, because recognised to have probable severe effects on human health or the environment (REACH Regulation, Article 57, pp. 141–42). Also, persistent, mobile, and toxic (PMT) chemicals are recognised for their pronounced polarity and environmental persistence (Fries & Sühring, 2023) and have been identified to represent a comparable level of concern to PBT/vPvB (Hale et al., 2020). Despite their potential risks, PMT plastic additives lack comprehensive regulation and thorough examination (Viljoen et al., 2023).

However, in most cases, substituting SVHC with other chemicals results in unregulated, often similar toxicity chemical choices (Brack et al., 2019). For food recycling, plastic and substituting with cardboard materials can also be considered a regrettable solution (Muncke, 2020). For example, substitutions of BPA with bisphenol S or F have been revealed to be of equal toxicity (Brack et al., 2019). Moreover, the substitution of DEHP with di(2-ethylhexyl) adipate (DEHA) is based on limited toxicological assessment and was found to migrate from PE (Gerassimidou et al., 2023). The migration of DEHA increases with fatty dairy products, and exposure to DEHA causes *in vivo* hepatic, neuro, and cardiac effects (Behairy et al., 2021).

However, most chemicals associated with plastic food packaging still lack toxicological characterisation, whether intentionally incorporated or unintentionally present (Muncke et al., 2020). Geueke et al. (2022, 2023) reviewed thousands of chemicals that can migrate from plastic FCMs, most of which are not listed and have no toxicological data available. Groh et al. (2021) reviewed 12.285 NIAS in FMCs, among which 1411 were of potential concern, and a third lacked toxicological data. According to Meng et al. (2023), only about one-tenth of chemicals present in plastic packaging present toxicological data. Notably, almost 40% of the chemicals authorised as monomers, plastic additives, or processing aids lacked any hazard classification (Wiesinger et al., 2021).

Muncke (2021) emphasises the necessity of inclusive hazard assessments for plastic packaging, including non-monotonic dose responses as seen with EDCs, as the assumption that low exposure levels represent

negligible risk, commonly applied in evaluating chemical risks for plastic food packaging, may not adequately protect human health (Muncke et al., 2022).

Characterisation of chemicals associated with plastic food packaging

Comprehensively identifying chemicals associated with packaging presents a series of challenges because of the material's multiplicity and the possible presence of NIAS, including TPs (Groh et al., 2019). Traditional monitoring methods are targeted analysis (TA), primarily focusing on identifying and quantifying individual chemical pollutants using reference standards to detect specific structures (Hinnenkamp et al., 2021). However, the increasing levels of emerging contaminants challenge the analytical identification. TA does not allow identifying unknown chemicals, such as TPs, as required reference standards (Tsochatzis et al., 2022).

Conversely, non-target analysis (NTA) allows for identifying known and unknown compounds, which also represents its challenge for the number of signals detected (Brunner et al., 2019). Moreover, it is also time-consuming and an expensive analysis (Hinnenkamp et al., 2021). Also, in the case of NTA, quantifying the identified chemicals requires analytical standards, which are only rarely available for chemicals of emerging concern (Neale et al., 2023).

While analytical approaches provide valuable information about the presence of specific contaminants, they often overlook the complex interactions and combined effects of environmental mixtures (Brack et al., 2019). To address the challenge posed by complex and unknown mixtures, bioassays or tests on cells can be employed to identify mixtures of toxicological concern. Indeed, bioassays have been recognised as a valuable tool in prioritising chemicals of concern based on effect-based thresholds (Muncke et al., 2023), a capability traditional analytical techniques lack (Neale et al., 2022).

Bioassays offer a unique advantage in assessing intricate low-level mixtures, even when the chemical composition is unknown, providing a more realistic representation of environmental exposure and directing chemical analysis and environmental monitoring (Severin et al., 2017; Brack et al., 2019; Zimmermann et al., 2019; De Baat et al., 2020; Groh et al., 2021; Neale et al., 2022). For instance, effect-based monitoring (EBM), an emerging approach that employs bioassays to identify areas of concern for investigative monitoring (Brack et al., 2019), has found applications in various matrices, including drinking water, recycled water, wastewater, and stormwater (Escher et al., 2018; Neale et al., 2023).

While bioassays provide insight into cellular-level effects, they serve as proxies for chronic effects (Neale et al., 2022), elucidating potential ecotoxicological and human risks. By using key characteristics (i.e. oxidative stress), *in vitro* assessment can elucidate the role of chemicals in key biochemical events, elucidating possible health outcomes and prioritising chemicals of toxicological concern (Muncke, 2021). The data obtained *in vitro* can be extended to *in vivo* through a process known as quantitative *in vitro*-to-*in vivo* extrapolation (QIVIVE) (Neale et

al., 2023). However, the existing testing methodologies only address some potentially significant effects (Brack et al., 2019).

The urgency for further research

Mixtures of chemicals in plastic packaging are often more critical than the polymers themselves (Groh et al., 2019; Gunaalan et al., 2020; Sewwandi et al., 2023; Meng et al., 2023) and require immediate research attention due to concerns about human exposure and health (Luo et al., 2022; Wojnowska-Baryła et al., 2022). Most consumer products can release hazardous chemicals, often unidentified (Zimmermann et al., 2019), and there is a need to understand these mixtures' environmental exposure and health effects (Muncke, 2021).

Current regulations focus on individual substances, overlooking mixture toxicity, non-intentionally added substances (NIAS), and low-dose effects (Groh et al., 2021). Risk assessment and monitoring strategies have shown minimal consideration for chemical release patterns, environmental concentrations, and potential adverse effects associated with FMC (Groh et al., 2021; Bridson et al., 2021). Additionally, the industry's lack of transparency regarding plastic packaging chemicals further complicates the issue (Groh et al., 2019).

In light of the potential harm associated with chemicals in plastic food packaging, urgent actions include evaluating the environmental pressure of the contamination, investigating the exposure, and assessing potential hazards of these chemicals to address existing data gaps and evaluate human health risks effectively (Groh et al., 2019; Muncke, 2020; Muncke, 2021; Geueke et al., 2022; da Costa et al., 2023; Fries & Sühring, 2023). Overall, these considerations underscore the necessity for our proposed research.

1.2 The overall aim of the proposal

In the realm of investigating the human health impacts of chemical mixtures concealed within plastic food packaging, this multidisciplinary research proposes a holistic approach to prioritise mixtures of concern and identify chemicals within those mixture to shed light on chemicals leaching from plastic food packaging. Samples will be collected via migration experiments and environmental samplings to account for multiple possibly contaminated sources. *In vitro* exposure and hazard assessments will be used to prioritise real-life samples and to extrapoate the relevance for human health. The mixtures that show a response *in vitro* will be characterised using non-target screening and *in silico* tools will be impled to predict the transformation product and try to unveil the complexity of the real-life mixtures. Overall, we aim to develop a framework to evaluate potential human health impacts from mixtures of chemicals found in plastic food packaging, with potential

applicability to the assessment of combined environmental exposure from other types of FCM or contaminants of environmental concern.

1.3 Research sub-questions

RESEARCH LINES 1 & 2

1) How can plastic food packaging-associated chemical mixtures be monitored for human health?
2) How can bioassays, analytical techniques and *in silico* tools be integrated into effect-based monitoring frameworks for detecting Substances of Very High Concern (SVHC)?
3) How can the framework account for the potential risks posed by transformation products, especially in cases where they exhibit different toxicological profiles than the parent compounds?
4) To what degree can the selected combined methods are reliable in identifying chemicals of toxicological concern? Are they replicable and guarantee statistically relevant results?

RESEARCH LINE 1

5) Which *in vitro* experiments can best monitor plastic food packaging associate chemicals' exposure and hazards?
6) To what extent can humans be exposed to mixtures of plastic packaging chemicals through ingestion and inhalation?
7) Which are relevant endpoints that can be assessed *in vitro*? Which endpoints remain uncovered?
8) Is there a toxicological concern associated with these mixtures that can migrate from plastic food packaging?
9) To what extent the selected methods are reliable and reproducible in assessing specific toxicological endpoints?
10) Which are the threshold concentrations for detecting toxicity of the selected bioassays?

11) Which is the reliability of the selected methods in assessing the combined exposure (food, water, and air)? Which is the reliability of hazard chemical indentification bioassays?

12) What key parameters significantly influence the developed framework's exposure and hazard identification accuracy, efficacy, and reliability?

RESEARCH LINE 2

13) What methods are suitable for simulating the migration of plastic packaging chemicals into food, water, and air?

14) Can plastic packaging chemicals migrate into different types of matrices, and how do various environmental conditions (e.g., temperature, UV radiation, microwave) affect this migration?

15) What limitations exist regarding accuracy, sensitivity, and practicality when conducting migration experiments for plastic packaging chemicals?

16) Which sampling strategy optimally monitors worst-case scenarios and controls spots for potential plastic packaging chemicals exposure?

17) What is the extent of contamination by plastic packaging-associated chemicals in air, water, and soil?

18) Does contamination increase near plastic production and recycling facilities?

19) Which strategy is suitable for identifying plastic packaging chemicals within expected low-level mixtures?

20) What specific chemicals compose the mixtures of concern identified earlier?

21) To what extent can computational methods identify unknown features within these mixtures?

22) Are there unidentified mixture? How many? What percentage of features can be identified compared to unidentified ones?

2.2 Approach

Given the scope of the topic, existing knowledge gaps, and the multidisciplinary expertise required, this proposal outlines the application for two PhD positions. The research plan is structured into six distinct phases, with each PhD candidate focusing in parallel on specific aspects of the project. A collaborative effort will be made to gather statistically significant results and develop an effect-based framework applicable to other chemicals of concern. Table 1 in the Appendix summarises the research activities (page 39).

2.1 Methodology

The research plan involves a multidisciplinary approach encompassing migration studies, *in vitro* exposure and hazard assessment to prioritise the characterisation of mixtures of concern. Bioassays and chemical analysis are complementary methods, offering unique insights and overcoming others' limitations (Neale et al., 2022). Chemical analysis consent identifies complex mixtures but may miss their toxicological effects. On the other hand, bioassays excel at pinpointing toxicological effects but do not identify the composition of the mixture.

RESEARCH LINE 1

Line 1 will focus on bioassay strategy development to assess *in vitro* the exposure and hazard to mixtures of chemicals associated with plastic food packaging and the translation to human health. The main objective is to prioritize chemical real-life mixtures likely migrated from plastic food packaging (collected in research line 2), using *in vitro* considerations of possible human exposure and related hazards.

Phase 1. *In vitro* strategy

Bioassays are a valuable approach for detecting the exposure and effects of mixtures, including unknown chemicals, that may migrate from FMC into food items (Severin et al., 2017). They can detect effects at low levels and shed light on the effects of mixtures when individual chemicals are present at concentrations below effect limits (Neale et al., 2023). Bioassays typically exhibit lower variability and faster results, require fewer sample volumes and are more cost-effective and ethical than *in vivo* ones. Moreover, they exhibit high sensitivity: for instance, some *in vitro* reporter gene assays can detect the effects of chemicals even when those chemicals are present at concentrations below the analytical limit of detection (Neale et al., 2023).

The essential equipment comprises cell cultivation and exposure incubation units, a biosafety cabinet to maintain a sterile cell culture environment, and a plate reader for quantifying the bioassay results (Neale et al.,

2023). In cases where validated bioassays are unavailable, suitable methodologies will be developed and implemented. A final overview of mixtures of concern migrated from plastic food packaging will be provided.

Step 1A. *In vitro* exposure strategy

As already highlighted, methods are needed to assess the impact of mixtures through various exposures and to compare these estimated impacts with environmental quality standards to safeguard both human health and the environment. As bioassays for detecting hazards do not consider ADME (Neale et al., 2023), *in vitro* models are implemented to assess the combined exposure via air, food and beverage consumption.

For food, water, and beverage exposure, ingestion cell assays will be performed. Commonly applied bioassays use colorectal cancer cell line Caco-2, which can differentiate into the most common cell type in the human intestine (Severin et al., 2017). *In vitro* intestinal cell humans such as InTESTine™ and Intestinal Explant Barrier Chip (IEBC) were previously used to demonstrate the passage of MNPs (Donkers et al., 2022). On the other side, to account for air contaminants in the lungs, *in vitro* lung human models can be used to study air pollutants, such as MucilAir™ used to demonstrate the passage of microplastics in simulated human intestines (Donkers et al., 2022).

Step 2A. *In vitro* hazard strategy

A high-throughput *in vitro* strategy to assess the effects of mixtures, as recommended by Escher et al. (2022), will be developed. Bioassays are valuable because they are endpoint-specific, allowing us to focus on specific modes of action (MoA) and their contribution to adverse outcome pathways (AOPs). AOPs provide simplified representations of biological interactions and mechanisms of toxicity at various levels of biological complexity, encompassing receptor-mediated endocrine impacts, genotoxicity, xenobiotic metabolic activation, stress response, and cytotoxic effects (Brack et al., 2019; Neale et al., 2023). Bioassays can also detect non-monotonic dose-response effects, such as those related to EDCs (Severin et al., 2017). When conducting these bioassays, one critical aspect is the potential need for metabolic activation, and it should be addressed within the experimental setup (Severin et al., 2017).

When assessing water quality, crucial toxicological endpoints include genotoxicity, carcinogenicity, reproductive and developmental toxicity, metabolic effects, endocrine disruption, and oxidative stress induction (Dingemans et al., 2018). Cytotoxicity is a proxy for acute systemic toxicity in humans and is an appropriate parameter for detecting sublethal levels in cell culture. Consequently, it should be conducted initially to establish the concentration range for subsequent experiments (Severin et al., 2017). While genotoxicity is the most commonly evaluated endpoint for water quality, it is essential to consider other potential hazards, such as neurotoxicity and immunotoxicity, when assessing plastic packaging (Muncke, 2021; Neale et al., 2022, 2023). A recent study by Meng et al. (2023) found that neurotoxicity was the most frequently detected toxicity among plastic packaging chemicals. Moreover, the limited routine consideration of endocrine disruption during the

authorization of chemicals in plastic food packaging is particularly concerning despite the known potential for these substances to exert such effects (Muncke, 2021).

CALUX® Reporter Gene Assays are receptor-specific, quantifiable, and highly sensitive specialised bioassays used to assess the activation of specific cellular receptors in response to exposure to chemical exposure (Severin et al., 2017). Reported-gene bioassays are more expensive than cell-based reporter gene bioassays, but they can elucidate key characteristics of multiple AOPs (Neale et al., 2023). A list of validated bioassays that emerged from preliminary analysis is reported in Table 2 in the Appendix (page 39).

Phase 2 - Bioassays development

Step 3A. Development of bioassays

The development of bioassays will be justified by the eventual lack of methodologies necessary during the strategy development (Steps 1, 2). For instance, as for the hazard assessment, from preliminary research, it emerged that endpoints such as reproduction, developmental, and neuro-toxicity still lack validated bioassays (Neale et al., 2023).

Step 4A. Validation of developed bioassays

New tests need validation, which could be done using chemicals with known toxicological effects, to demonstrate the methodology's accuracy, precision, repeatability, inter-assay variation and reproducibility, as Ederveen (2010) suggested. Moreover, linearity shall be demonstrated, usually with a minimum of five concentrations, to demonstrate the test's capacity to produce results that directly correlate with the substance concentration within a defined range (Ederveen, 2010).

Phase 3. *In vitro* assessment of mixtures

The samples collected in phase 1, research line 2 (see page 14) will be assessed *in vitro* to consider inhalation and ingestion exposure and related hazards and prioritise mixtures of concern, that will undergone chemical characterisation in phase 4, research line 2.

Step 5A. *In vitro* exposure assessment

The whole-mixture samples previously collected will be tested *in vitro* according to the strategy developed (Step 1), to understand the human exposure, thus select chemicals among these mixtures that can be uptaken by human-specific cells, accounting for inhalation and ingestion exposure.

Step 6. *In vitro* hazard assessment

A series of bioassays that emerged from the strategy development (Step 2) will be done on the whole-mixture sample previously collected to assess relevant toxicological endpoints, including the ones selected by the preliminary analysis (see Appendix, Table 2, page 40). Bioassays can also elucidate the concentrations of mixtures able to exert a specific effect by using different dilution samples because the effect detected is proportional to the concentration (Neale et al., 2023). To assure the quality of the analysis, a positive reference compound, a negative control, a solvent control, and blank samples must be integrated into regular bioanalytical procedures (Neale et al., 2023). IAS recognised as being able to migrate (i.e. BPA and PAEs) could serve as a positive control to be run at different concentrations to derive the effective concentrations (Neale et al., 2023). Moreover, intraplate, intraassay, and interassay replications should be done (Neale et al., 2023).

Step 7A. Identify effect-based trigger values

Effect-based trigger values are associated explicitly with the bioassay used and the endpoint considered; therefore, they shall be implemented when necessary (Neale et al., 2023). EBTs represent the highest permissible concentrations established concerning human health or ecological risk considerations and are specific for the type of matrices in analysis (Neale et al., 2023). Identifying correct effect-based trigger values is essential to interpret *in vitro* results adequately and suggest precautionary but not too conservative values (Dingemans et al., 2018). Moreover, developing EBTs has the advantage of making the bioassays replicable for monitoring; therefore, EBTs shall be included in the framework for effect-based monitoring.

EBTs are typically expressed as bioanalytical equivalent concentrations (BEQ), the concentrations of a benchmark substance that produces an equivalent toxicological effect to that induced by the chemical combination present in a sample (Neale et al., 2023). BEQ can be derived using *read-across* from existing Environmental Quality Standards (EQS) values for single chemicals (Escher et al., 2018) and allow for the comparison of findings across various bioassays, samples, and research studies, including chemical analysis (Neale et al., 2023). However, this approach does not require EQS for all the mixture components in the analysis (Brack et al., 2019).

Step 8A. Overview of hazardous mixtures

Overview of mixtures found to be of concern according to the exposure and hazard *in vitro* assessment (Steps 5 and 6). At this stage, considerations on the transferability of the bioassay results to human assessment have not yet been done. However, mixtures are here prioritised for the chemical analysis of Phase 3, research line 2, as associated with some toxicological effects in organisms.

Phase 4. Human extrapolation

Step 9A. QIVIVE extrapolation

Quantitative *in vitro*-to-*in vivo* extrapolation (QIVIVE) is necessary to translate the *in vitro* results in humans. After generating a concentration-response relationship *in vitro*, it is possible to predict an *in vitro*-based point of departure (PoD) (Paini et al., 2019). It is worth noting that the PoDs considerations, often used for NIAS, do not apply to substances classified as CMR or PBT (Geueke et al., 2013). Physiologically-based kinetic (PBK) models use differential equations to describe the ADME in an organism. They can translate the *in vitro* EC obtained into corresponding human oral doses *in vivo* and establish the relevance for *in vivo* scenarios, deriving safe chemical intake levels (Paini et al., 2019).

Phases 5 and 6 are collaborative and will be described at the end of line 2 (see page 22).

RESEARCH LINE 2

This research line will focus on the collection of samples from both migration experiments to food and beverages in critical conditions and environmental contamination. Moreover, chemical identification using non-target screening and computational methods to identify unknowns is used to characterise the mixtures prioritised by research line 1.

Phase 1. Sampling strategy

Various commonly used plastic food packaging materials will undergo migration experiments under different critical environmental conditions, with food and beverages they are recommended for by design. Moreover, the possible leach to environmental matrices will be considered because not only can FCMs release chemicals with the products they are in contact with, but they also can contaminate the environment when they are recycled, landfilled, incinerated or littered (Groh et al., 2019).

Step 1B. Migration experiments strategy

Food and beverage migration experiments will be made to evaluate the migration of chemicals from different types of plastic food packaging, including recycled plastic, as seen of concern (Geueke et al., 2023). Literature research and strategic planning will be done to define a suitable experimental approach to consider migration to food and beverage, water, air, and soil samples. Migration, extraction, and leaching experiments applied to plastic additives have been reviewed by Bridson et al. (2021).

Step 2B. Environmental sampling strategy

The environmental sampling strategy for collecting real-life mixtures, as suggested by Escher et al. 2022, will be the object of research, as plastic food packaging associated chemicals can migrate to water (Gunaalan et al. 2020), air (Maceira et al., 2019), and soil (Viljoen et al., 2023). Attention will be paid to soil, water, and air in the proximity of recycling facilities, landfills, and incinerators, where contamination is expected. Indeed, Maceira et al. (2019) found plastic additives in outdoor air particulate matter in Europe.

The sampling campaign will last approximately 8-12 months. The sampling methodology will be designed according to existing literature and using the EPA's Trade-off Tool for Sampling (TOTS) https://tots.epa.gov/, an online application designed to facilitate the creation of sampling designs visually and the consideration of cost-benefit evaluations, different sampling techniques, and the extent of sampling coverage.

Locations will be evaluated among urban, suburban, and industrial areas present in the Netherlands and selected based on their proximity to potential pollution sources. Locations of attention include a) worst-case scenarios such as plastic recycling facilities and plastic food packaging production companies, i.e. Berlin Packaging Netherlands B.V. in Nijmegen, and b) control location control in a park in a city (i.e. Amsterdam).

Evaluations of the sampling strategy will be done specifically for each environmental matrix. For example, for wastewater, composite sampling over 24 hours is suggested by Neale et al. (2023) to consider variation over time, while for drinking water, grab sampling can be sufficiently informative (Neale et al., 2023). However, passive sampling gives information on more significant volumes of water (Neale et al., 2023).

Moreover, the research could use Environmental Distribution models to assess the availability of chemicals in environmental compartments to predict where to search for expected levels of identified plastic packaging chemicals. Schwarz et al. (2023) applied the Material Flow Analysis framework to calculate and assess the worldwide release of plastic pollutants from the plastic value chain into various environmental compartments.

Phase 2 - Samples collection

Step 3B. Food and beverages migration experiments

Following the strategy developed in Step 1, migration experiments will be conducted. Various conditions will be applied, including high temperatures, stressors to simulate ageing, UV treatments, dishwashing, and microwave exposure, as found to be critical conditions for the migration (Can & Yerlikaya, 2019; Luo et al., 2022; Sewwandi et al., 2023; Gerassimidou et al., 2023; Geueke et al., 2023). The experiments will use different food samples because fatty food facilitates the migration of lipophilic chemicals (Sewwandi et al., 2023). Moreover, migrations can be performed using the cellular culture medium as an aqueous simulant, as suggested by Maisanaba et al., 2014.

Step 4B. Environmental sample collection

Following the strategy developed in step 2, a sampling campaign will be done in the Netherlands, accounting for these preliminary considerations.

(a) The air sampling could use the car of the Project Air View associated with passive sampling, as it indicates temporal variation.

(b) Analysis of surface water, groundwater, wastewater, and drinking water shall involve at least two or three samples for each location to account for variability and errors (Neale et al., 2023).

(c) Samples will be taken at different depths to assess the vertical distribution of mobile chemicals in soil and sediments (Viljoen et al., 2023).

The environmental samples will also be pre-treated before being analysed in vitro in phase 3 of the research line 1 (see page 20). Pretreatment is suggested to enrich the samples to make the signal to the bioassay detectable (Brack et al., 2019). Commonly used pre-treatments before bioassay analysis for water samples are solid-phase extraction (SPE) or passive sampling followed by liquid-liquid extraction methods. However, they extract only organic chemicals (Neale et al., 2023), excluding chemicals that might be relevant for this research. Neale et al. (2023) suggest that the risk of losing chemicals in the extraction is compensated by reducing possible confounders. Moreover, without enrichment, the effect in the original sample might be too low to be detected; thus, it is suggested that chemicals are expected to be in low concentrations (Neale et al., 2023).

Phase 3 - Suspect list of chemicals of concern

Step 5B. Suspect list

The suspect list of chemicals associated with plastic food packaging will be developed over the research. Considerations include: (a) identified human hazards, exposure or risk concerns in the literature, i.e. inclusion in one of the alert lists developed by Wiesinger et al. (2021), Groh et al. (2019, 2021), Geueke et al. (2023), and Meng et al. (2023), (b) inclusion in the Union List of authorised chemicals for FCMs, (c) inclusion in the list of 235 (to date) substances in the Candidate List of SVCH (REACH, Annex III).

Step 6B. Prediction of transformation products

In silico models such as Chemical Transformation Simulator (CTS), EAWAG-BBD UM Pathway Prediction System, enviPath, and BioTransformer can help to predict the formation of transformation of given compounds due to biotic reactions, i.e. environmental microbial transformation, and abiotic reactions such as abiotic hydrolysis and abiotic photolysis (Brunner et al., 2020). By predicting possible TPs, a suspect screening list can guide their analytical identification (Boyce et al., 2023).

(plus) Step 7B. *In silico* prioritisation of transformation products

As the amount of prediction products can be vast, prioritization could be made based on (a) the occurrence of prediction and (b) predicted hazard alerts. *In silico* models like QSARToolbox and VEGAHub tools can predict hazard alerts. The endpoints better evaluated *in silico* are skin corrosion and irritation, eye corrosion, genotoxicity, carcinogenicity, and cytotoxicity (OECD, 2018), and eventual prioritisation might be necessary and made accordingly. However, selecting unknown compounds based on *read-across* evaluation might exclude chemicals of concern for the absence of data used by the algorithm to derive hazard alerts.

Phase 4 - Chemical analysis of hazardous mixtures

The mixtures of concern prioritised by the *in vitro* assessment in phase 3 of line 1 will be qualitatively analysed using NTA associated with predictive tools to identify chemicals of concern as a recommended methodology for regulatory monitoring (Lai et al., 2021), using the developed suspect list accounting for TPs.

Step 8B. Analytical detection of chemicals of concern

While the qualitative identification of NIAS is possible using analytical techniques, quantitative identification is difficult due to the lack of analytical grade standards - needed to confirm the tentative detection (Tsochatzis et al., 2022). The most common analytical techniques to analyse chemicals that migrate from FCM are liquid (LC, HPLC, UHPLC) or gas (GC) chromatography - used to separate the diverse components of complex matrices - coupled with high-resolution mass spectrometry (HR-MS) - to identify the different signals (Tsochatzis et al., 2022). Examples of NTA that successfully identified unknown chemicals are high-resolution tandem mass spectrometry (HRMS/MS) or multi-step pyrolysis coupled with a gas chromatography-mass spectrometer (pyr-GC/MS), already used to identify chemicals possibly released in the air from plastic products (Brunner et al., 2020; Even et al., 2019; Akoueson et al., 2021).

One main challenge of NTS is the interpretation of the unknown signals detected (Zimmermann et al., 2019), and the combination with *in silico* tools could help in this regard. *In silico* tools can help relate unknown signals of the NTA with the transformation of the plastic additive of origin by predicting fragmentation spectra of unknowns (Lai et al., 2021; Tsochatzis et al., 2022). CFM-ID v4.0.0 can be used to predict the fragmentation spectrum of the predicted TPs (Step 2) to be compared with the signals of the NTA (Wang et al., 2021).

(plus) Step 9B. Fractionation of mixtures

Whether the amount of signals detected is too extensive for a mixture, eventual fractionation of the migration samples to narrow the attention to chemical fractions responsible for the biological effect can be done, as the effect-directed strategy proposed by Rosenmai et al. (2017) for FCMs. Effect-directed analysis (EDA) is a powerful approach for prioritising mixtures of concern. It involves a series of steps, including hazard

identification through *in vitro* bioassays, physicochemical fractionation of the mixture for analysis, and chemical analysis of the fraction of interest. This systematic approach narrows down the compounds responsible for observed effects (Rosenmai et al., 2017).

LINE 1 & 2

Phase 5. Results

Step 10. Statistical analysis

Statistical methods shall be considered throughout the entire research line to ensure statistically relevant results. For example, interference analyses compare the migration levels of different plastic packaging materials, and analysis of variance (i.e. ANOVA) and t-tests to compare migration levels under different conditions. Moreover, statistical analysis allows for identifying outliers. Statistical analysis will be conducted in consultation with the Utrecht University methodology and statistics training and support to ensure the appropriate application of statistical methods.

Step 11. Overview of chemicals of concern migrated from plastic food packaging

A collaborative effort will be made to create an overview of identified chemicals and unknown features found to migrate from plastic food packaging found into food, beverages, and environmental matrices by unifying the information gathered through the two PhD lines. Specific aspects concerning the migration will be elucidated, including the factors highlited by Weber et al., (2023): 1) the type of plastic material responsible for their migration, 2) the physicochemical characteristics of the migrated chemicals, 3) the type of matrix involved in the migration, and 4) the conitions at which these mixtures are released. Moreover, the exposure and hazards detected concerns related to the mixtures containing these chemicals will be provided.

Phase 6 - Effect-based framework

A framework for effect-based monitoring applicable to other chemicals of concern will be provided. Gathering the expertise of both researchers, an effect-based monitoring framework will be developed to assess combined exposure via air, water, and food and relative human health hazards. Moreover, the framework could indicate how to define SVHC, inform risk analysis for product development and promote the development of safe materials and final products aligned with SSbD goals. Conceptualised risk assessment framework for making decisions on the replacement.

Step 12. Framework development

A comprehensive framework will be developed, integrating diverse analytical, *in silico*, and *in vitro* methodologies to predict environmental monitoring of substances of toxicological concern.

2.2 Duration of the research

The present research will last four years (48 months), and imply two research positions and two technicians to support the chemical analysis and bioassays development. In Table 1 a temporal division of the two research lines is available in the Appendix.

2.3 Laboratory equipment

The present research proposal aligns with the expertise already available at KWR, TNO, IRAS at Utrecht University and the University of Maastricht laboratories, which includes experienced researchers in environmental chemistry, analytical techniques, bioassay development, *in silico* predictions, exposure and hazard assessment.

- Food Packaging Migration Experiments - Circular Economy and Environment Research Group, TNO / the University of Maastricht
- Non-Target Screening - Laboratory for Materials Research and Chemical Analysis, KWR
- *In silico* prediction of transformation products - Chemical Water Quality and Health team, KWR
- Exposure Assessment - Circular Economy and Environment Research Group, TNO, RAPID Projects
- Air Sampling and Chemical Analysis - Circular Economy and Environment Research Group, TNO / Project Air View, Utrecht University
- Water Sampling, Sediment Sampling and Chemical Analysis - Laboratory for Materials Research and Chemical Analysis, KWR
- Hazard assessment - Chemical Water Quality and Health team, KWR / IRAS, UU

2.4 Supervision

Supervisors working at KWR, TNO, Utrecht University, and Maastricht University will be involved in the research. Table 3 collects the researchers and their expertise at disposal for this research.

Table 3. Overview of supervision and expertise available for each research phase as a collaboration of TNO, Water Research Institute (KWR), Institute for Risk Assessment Sciences (IRAS) at Utrecht University (UU), and Maastricht University (UM).

	Activity	Institution	Person	Expertise
PhD 1	Exposure assessment	TNO-IRAS	Technician 1 Ingeborg Kooter (TNO)	Exposure assessment
	Hazard assessment	TNO-KWR-IRAS	Technician 1 Ingebork Kooter (TNO) Milou Dingemans (KWR)	Hazard assessment
PhD 2	Migration experiments	UM TNO	Misha Vrolijk (UM) Ingeborg Kooter (TNO)	Food contact materials
	Environmental contamination	KWR-IRAS-TNO	Technician 2 Gerard Hoek (UU)	Sampling strategy
	Chemical analysis	TNO-KWR	Technician 2 Astrid Reus (KWR)	Non-target screening *In silico* tools

2.5 Research deliverables

1) Overview of migrated chemicals' mixtures from plastic food packaging able to be uptaken by humans and exert a toxicological effect, specifying the plastic packaging and the experimental conditions associated, and the exposure route and MoAs involved.
2) Overview of chemicals identified in mixtures of concern listed in Overview 1, including known chemicals for which quantification is possible due to analytical standards and chemicals for which only a tentative identification is possible.
3) Overview of environmental detection of listed chemicals in Overview 2.
4) Framework for effect-based monitoring of chemicals of concern, applicable to other FCMs.
5) At least four publishable papers open access to share the results highlighted in Overviews 1-3 and the developed framework.
6) At least once every six months, a lay article to share online and share the present research's intentions, methodologies, and results.

3. Risk Assessment

While the outlined methodologies and approaches hold promises to apply a holistic approach to evaluating plastic food packaging-associated chemical mixtures' health impacts, several challenges and potential risks must be acknowledged. The applied methods might present challenges and need to be more predictable. Flexibility in adapting the research plan based on emerging challenges is needed to meet the research goals.

Migration studies. The risk is that the experimental conditions will not be realistic enough to simulate all the possible environmental stressors (i.e. meteorological conditions) the plastic food packaging might find.

Environmental sampling. Environmental sampling collection is done to account for real-life mixtures. However, this increases the risk of confounders, as the effect of combinations present in the environment is likely a consequence of different sources of exposure, not just food packaging. However, suppose chemicals associated with plastic packaging are found in mixtures of concern. In that case, they contribute to an effect, even though that is also due to other micropollutants not included in food packaging. Obtaining precise data on contamination levels near specific facilities might be challenging, and proving direct causation between contamination and facilities can be complex. Moreover, environmental sampling can be subject to variability due to spatial and temporal variations. Adopting a robust sampling strategy, ensuring replicates, and utilising statistical methods can help mitigate the effects of variability.

Analytical challenges. The detection and quantification of unknown complex mixtures, such as the ones expected to migrate from plastic food packaging, can be challenging due to the low concentrations and potential interference from other compounds. The number of unknown features can be reduced using predictive tools for transformation products. However, the absence of analytical standards can prevent the quantification of such unknown signals.

Predictive tools. Innovative analytical techniques combined with *in silico* tools are still a development method. Existing tools account for different reaction pathways. However, more tools are needed to increase comprehensivity. This might result in excessive predicted chemical structures, and a prioritisation step might be required (Step 7). However, the prioritisation step entails the risk of excluding chemicals of concern not identified *in silico*.

Bioassays development. While bioassays are innovative tools for accounting for the hazards related to low levels of chemical mixtures, they are a field in development, and crucial endpoints still need validated tests. Developing suitable positive and negative controls, comparing bioassay results with chemical analysis, and employing multiple bioassays could enhance their reliability. Developing new methods is a long process, which might not be conducted in this research.

Translation to human health. Using *in vitro* models for exposure assessment poses the risk of a lack of biological relevance. Therefore, the *in vitro* tests shall be appropriately validated using known chemicals for which the comparison with *in vivo* data is possible.

Transformation products' identification. Identifying and characterising transformation products resulting from environmental degradation is complex. Using *in silico* tools can facilitate the identification of unknown features but are subject to model uncertainties and potential limitations. Using different models and validating them by comparing experimental data can enhance their reliability.

Quantitative analysis. While tentative identification is possible, quantitative analysis requires analytical standards that are only sometimes available. However, the qualitative research remains informative for future monitoring efforts., even though it will not be possible to translate that information into human exposure levels.

Data integration. Integrating data from different sources (chemical analysis, bioassays, *in silico* predictions) to develop comprehensive risk assessment frameworks can be complex. Developing appropriate algorithms and statistical models for data integration will be necessary, and proper support must be assured.

Time and resource management. The research involves numerous steps and different research groups; thus, planning various expertise contributions will require thorough organisation.

Regulatory purposes. Developing risk assessment frameworks aligned with regulatory requirements and standards is crucial for the applicability of research outcomes. Therefore, the research shall be aligned with regulatory provisions from its onset.

4. Scientific and societal impact

This study aims to investigate using bioassays to prioritise and detect chemical mixtures of concern possibly released from plastic food packaging. With the combination of chemical and biotesting methodologies, the research might elucidate the impact of such products on human health and the environment. The proposed study holds short- and long-term implications for environmental science and toxicology, which extend to regulatory policy, public health, and environmental monitoring and mitigation.

4.1 Scientific impact

Short-term impacts

Improved safety assessment. By conducting *in vitro* assessments and non-target screening, our research will enable the identification of previously unknown chemical compounds within plastic food packaging. This will enhance our ability to assess food contact materials' safety comprehensively. This research can contribute to determining the release of chemicals from plastic food packaging into food and beverages and environmental matrices (air, water, soil, and sediments). Investigating substances for which toxicological information is still lacking (as highlighted by the literature) could bridge the gap between product chemicals and their possible migration, human uptake, and related toxicological concerns. Overall, the results can offer valuable assessments of chemicals released from plastic food packaging potential exposure and hazards to inform health-related risks. This research addresses the pressing need for a deeper understanding of chemical migration, with direct applications in regulatory compliance and consumer safety.

Prioritisation of chemicals of concern. With the myriad of chemicals possibly migrating from plastic food packaging, prioritisation is needed. Our effect-directed evaluation approach will provide a structured framework for prioritising chemical mixtures that warrant closer scrutiny. This effect-based approach will facilitate more efficient and practical research in identifying and mitigating risks associated with food packaging. Indeed, this research has the potential to identify NIAS released from plastic food packaging that require attention. Moreover, this research can provide a holistic understanding of the distribution in the Netherlands of chemicals released from plastic food packaging, enhancing our understanding of environmental contamination.

Long-term impacts

Monitoring alerts. By harmonising analytical and bioassay methodologies into a framework, the research aims to guide future monitoring strategies to tackle chemicals of toxicological concern.

Further investigating the experimental conditions influencing chemical migration from plastics can guide research on materials and developing safer and more environmentally friendly food packaging materials. These methodologies extend beyond plastic food packaging, offering potential applications in monitoring chemicals of concern in various plastic products and food contact materials (i.e. board and paper, for which concern is expressed).

Environmental conservation. Our investigation into the extent of contamination near recycling facilities and environmental compartments will contribute to understanding plastic pollution and its consequences, informing strategies for mitigating plastic-related risks. Moreover, by understanding the distribution of the investigated chemicals in the environment, this research could inform environmental sciences about the distribution patterns of chemicals of concern in the environment.

Regulatory evolution. This research might contribute to a deeper understanding of the complexities of environmental contamination and direct future developments and mitigation strategies. By shedding light on the environmental consequences of plastic food packaging, our work can inspire sustainable practices and policies to reduce plastic pollution and be more protective of human health. The findings from our research can inform regulatory bodies and policymakers about the potential risks associated with specific types of plastic packaging and their use in various conditions, leading to evidence-based regulations to protect consumers. The findings could contribute to creating guidelines to establish criteria for plastic food packaging.

Identify future research directions. The research can help identify robust methodologies and aspects needing improvements.

4.1 Societal impact

By monitoring human health's possible exposure and hazards to chemicals released from plastic food packaging, the research can contribute to safeguarding the environment and human health. This research can induce consumer awareness, inform environmental monitoring and mitigation activities, and inform future regulatory decisions, reflecting a commitment to safeguarding public well-being.

Short-term

Identifying chemicals of concern. This research has the potential to uncover previously unidentified chemicals of concern, shedding light on the adverse effects of unsustainable consumer products, particularly within the predominantly single-use market of plastic food packaging. By investigating the ingestion and inhalation routes related to plastic food packaging chemicals, our research may reveal the negative consequences of their usage for human health and the extent of environmental contamination. By assessing human exposure levels and associated hazards, our research can inform public health initiatives and raise awareness about the potential health risks of chemicals leaching from plastic food packaging.

Investigating recycling. Our research also delves into environmental contamination linked to recycling facilities, highlighting that recycling alone is not the ultimate solution. Instead, it underscores the importance of waste reduction as a primary step in addressing environmental challenges.

Public awareness. Our research is dedicated to enhancing public awareness concerning the potential health risks associated with plastic food packaging products. We firmly believe that every individual has the right to be informed about the products they use and the potential risks these products may pose to their health. To ensure widespread dissemination of our research findings, we are committed to presenting our results through easily understandable lay articles included in our research deliverables. By raising public awareness about the environmental and health implications of chemicals associated with plastic food packaging, the research can highlight the need for reducing exposure and encourage more informed consumer choices, potentially reducing the demand for plastic-packaged food products.

Long-term

Environmental monitoring and mitigation. A central focus of our research is understanding how these chemicals disperse within the Dutch environment and identifying areas of concern. This knowledge will empower informed decision-making for environmental mitigation and decontamination strategies, focusing on pinpointing regions with existing contamination.

Future regulatory impact. By sharing our research findings in an accessible manner with various stakeholders, including government agencies, industry representatives, and non-governmental organisations (NGOs), we aim to inspire the refinement of international regulatory frameworks and guidelines. This collaborative effort can lead to the development of more comprehensive environmental protection strategies.

Promoting safer products. Moreover, the methodologies developed during this study possess the potential to pave the way for the creation of safer products. As our understanding of chemical migration and exposure risks grows, the food packaging industry can use this knowledge to innovate and produce safer packaging materials for consumers and the environment. These methodologies align with the principles of the Safe and Sustainable by Design concept, catalysing responsible product design and manufacturing practices.

5. Ethical considerations

Environmental responsibility. The research must consider and limit as much as possible the potential environmental impact of sampling operations to ensure that the techniques implied are environmentally responsible. To that end, we will conscientiously select sampling locations, taking great care to minimise any potential disruption to the delicate balance of natural habitats and sensitive ecosystems. By doing so, we acknowledge our ethical duty to protect and preserve the environment in which we conduct our research.

Use of materials. We acknowledge that the research process involves the use of laboratory materials that can have a notable environmental footprint. In pursuing ethical research practices, we are resolute in mitigating this impact. Our approach centres on the conscientious selection and utilisation of eco-friendly sampling materials to minimise significant waste generation. This conscientious choice aligns with ethical standards and underscores our commitment to minimising our research's ecological footprint.

Reduce animal testing. By advancing the use of New Approach Methodologies (NAMs), the research on animals can be reduced, which aligns with a more ethical and responsible way to provide research. By employing *in vitro* models and computational predictions, the study aims to minimise the use of animals in toxicity assessments. This approach aligns with ethical principles that prioritise the reduction, refinement, and replacement of animal testing (3Rs).

Travel considerations. In our commitment to reducing our carbon footprint, we will adopt a considerate approach by judiciously limiting our conference participation. This prudent decision is motivated by the desire to minimise unnecessary travel-related emissions. In contrast, this research will invest in the sharing of results via digital methods.

Sharing of results. According to the EU Transparency and Openness Promotion guidelines, results will be shared as open access. Researchers are ethically obligated to communicate their findings to the public, policymakers, and relevant stakeholders. By adopting this approach, we aim to foster collaborative efforts and ensure the broad distribution of data, thereby enriching public awareness and informing regulatory decisions.

Responsible use of findings. Our research holds the potential to influence industry practices, regulatory frameworks, and the safety of future generations. In light of these significant implications, we emphasise our ethical duty to consider the consequences of our work carefully. Our responsibility is to advocate for ethical and responsible practices within the food packaging industry. By doing so, we aim to ensure that the impact of our research on society is grounded in positive responsible and sustainable practices.

Conflict of interest. Maintaining the integrity and impartiality of our research is of utmost importance. Regardless of potential conflicts of interest, we commit to conducting our research objectively and transparently. We understand the ethical necessity of disclosing any potential conflicts to ensure the credibility

of our research outcomes. For this reason, any agreement with the industry sector will be made, guaranteeing impartiality of the assessment of plastic food packaging products.

Adherence to research standards. Our commitment to ethical research extends to adherence to recognised research standards. We pledge to follow the Netherlands Code of Conduct for Research Integrity adopted by NWO. This code embodies the fundamental principles of honesty, scrupulousness, transparency, independence, and responsibility, which guide our research endeavours and ensure the highest ethical standards are upheld throughout our work.

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APPENDIX

Table 1. Activities for the research proposal on plastic-associated chemicals' mixtures evaluation are divided into two parallel research lines accounting for six phases and 12 activities among which the last three of collaborative efforts.

Line 1					Line 2			
Phase 1	*In vitro* strategy	Step 1A	months 1-2	*In vitro* exposure strategy	Sampling strategy	Step 1B	months 1-2	Migration experiments strategy
		Step 2A	months 3-4	*In vitro* hazard strategy		Step 2B	months 3-4	Environmental sampling strategy
Phase 2	Bioassays development	Step 3A	months 5-12	Development of bioassays	Samples collection	Step 3B	months 5-12	Food and beverage migration experiments
		Step 4A	months 12-16	Validation of developed bioassays		Step 4B	months 12-16	Environmental sampling
Phase 3	*In vitro* assessment of mixtures	Step 5A	months 17-23	*In vitro* exposure assessment	Suspect list of chemicals of concern	Step 5B	months 17-18	Suspect list
		Step 6A	months 24-30	*In vitro* hazard assessment		Step 6B	months 19-24	Prediction of TPs spectra
		Step 7A	months 26-32	Identify effect-based trigger values		Step 7B	months 25-30	*In silico* prioritization of TPs
		Step 8A	Months 33-35	Overview of hazardous mixtures				
Phase 4	Human extrapolation	Step 9A	months 36-41	QIVIVE extrapolation	Chemical analysis of hazardous mixtures	Step 8B	months 30-36	Analytical detection of chemicals of concern
						Step 9B	months 36-41	Fractionation of mixtures
Line 1 & 2								
Phase 5	Results	Step 10	months	Statistical analysis through the whole research				
		Step 11	months 42-45	Overview of chemicals of concern migrated from plastic food packaging				
Phase 6	Framework development	Step 12	months 45-48	Effect-based framework				

Table 2. Available validated bioassays for cytotoxicity, oxidative stress, genotoxicity, carcinogenicity and endocrine disruption endpoints, among which some were used for plastic FCMs.

endpoint	MoA	bioassay	process/advantages	cell line	used for FCMs evaluation	reference
Cytotoxicity	Cell toxicity	RNA synthesis inhibition	Inhibition of the synthesis of RNA	HelaS3 cell line Caco-2 cell line	PET, paper/board, adhesives	Severin et al., 2017
Oxidative stress	nuclear factor (erythroid-derived 2)-like 2 (Nrf2) mediated oxidative stress	AREc32 reporter gene assay	Nrf2 mediated oxidative stress response	human breast cancer cell line MCF7	-	Escher et al., 2012
		Nrf2- CALUX	reporter gene assay	human U2OS cell line	-	Van Der Linden et al., 2014
Genotoxicity	damage to the nucleus	Ames fluctuation test	bacterial reverse mutation OECD TG 471	S. typhimurium /E. coli	PET, paper/board	Severin et al., 2017
		Comet assay	Induced mechanic DNA damage	HL-60 mammalian cells	PET, PP, paper/board	Severin et al., 2017
		p53 CALUX	reporter gene assay	human U2OS cell line	-	Van Der Linder et al., 2014
		Micronucleus assay	Induction of chromosomal damage	CHO cell line	PET	Severin et al., 2017
Carcinogenicity	PPARγ expression	PPARγ-CALUX	Within AOPs of numerous diseases	human cell lines	-	Piersma et al., 2013
Endocrine disruption	Aryl hydrocarbon receptor (AhR)	DR-CALUX	Dioxin receptor endogenously	H4IIE liver human cells	-	Piersma et al., 2013
		AhR-CALUX	reporter gene assay	H4IIE rat hepatoma cells	recycled paper	Severin et al., 2017
	Androgen receptor (AR)	AR-CALUX	reporter gene assay	human cell lines	PET, PP, PE, PS	Piersma et al., 2013; Severin et al., 2017
		Yeast Androgen Screen (YAS)	reporter gene assay	human androgen receptor	PET, PP, PE, PS, paper	Severin et al., 2017
	Estrogen receptor (ER)	Yeast Estrogen Screen (YES)	reporter gene assay	human estrogen receptor	PET, PP, PE, PS, paper	Severin et al., 2017
		ER-CALUX	Account for receptor interactions	Human T47D cell line	PET	Piersma et al., 2013; Severin et al., 2017