

Partnership for the Assessment of Risks from Chemicals

Deliverable D6.3

1st report on innovative approaches for real-life mixture risk assessment

WP 6 – T6.2



Partnership
FOR THE
Assessment
OF
Risks
FROM
Chemicals



Co-funded by
the European Union

This partnership has received funding from the European Union's Horizon Europe research and innovation programme under Grant Agreement No 101057014.

Technical reference	
Work package	WP6 – Innovation in regulatory risk assessment
Task	T6.2 – Integrative exposure and risk assessment
Dissemination level ¹	PU = Public
Lead Beneficiary/ Responsible AE	RIVM and ANSES
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Due date of deliverable	30 April 2025
Actual submission date	19 June 2025

¹ PU = Public

² WP/task co-leader(s) from the same WP

³ Independent Reviewers that should not be part of the task related to the AD/D or part of projects related to the Task

Document history

Version	Date	Reviewer name/Institutions	Short description of changes
1	28 April 2025	Authors and co-authors	First compiled version
2	21 May 2025	Reviewers of the deliverable or additional deliverable	Draft version with comments provided by reviewers
3	05 June 2025	Authors	Draft version with answers to the reviewers' comments
4	18 June 2025	Authors	Cleaned version

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Abstract

Human populations are exposed to chemical mixtures on a continuous basis via several sources and routes of exposure. The Partnership for the Assessment of Risks from Chemicals (PARC) Real-life mixtures project has proposed a strategy to perform mixture risk assessment (MRA) of chemicals based on human biomonitoring (HBM) data. Four case studies were established based on PARC prioritised substances and effects, namely: 1) Pesticides – Neurotoxicity; 2) PFAS - Immunotoxicity; 3) Metals – Nephrotoxicity; 4) Metals – Developmental neurotoxicity. HBM data from 14 countries across Europe, was gathered, harmonised and used in the case studies. Hazard data, such as Human Biomonitoring-Guidance Values (HBM-GV), Human Biomonitoring-Toxicological Values, No-Observed-Adverse-Effect-Levels (NOAEL) and Relative Potency Factors (RPF), were collected or established for their application in the MRA. Toxicokinetic data were also collected and have been used in the Pesticides – Neurotoxicity case study to perform reverse dosimetry. Based on the hazard availability data, different MRA approaches were applied (Margin of Exposure Total, RPFs, and modified Reference Point Index). MRA analysis was performed in the Monte Carlo Risk Assessment software, which is part of the PARC model network. The results of the case studies can be considered as proof of concept for the first-tier MRA strategy developed in the PARC Real-life mixtures project. The findings revealed a potential concern regarding exposure to chemical mixtures in several European populations, since the threshold of negligible concern was often exceeded. Within the case studies, the risk drivers of the mixtures were identified. Several uncertainties and challenges were highlighted, along with proposed solutions for refinement in a second-tier MRA. Furthermore, the strategy will be extended to mixtures crossing regulatory silos identified from co-exposure to the different chemical groups. This work will continue to be conducted in synergy with European agencies and other European research projects. This work is a proof-of-concept, however it needs further discussion on the data and methodology used in order to consolidate possible use of HBM data for risk assessment. At this stage it wasn't possible to harmonise the uncertainty analyses over case studies and to explore in depth how uncertainties may impact on the results. This is partly due to the complexity of the case studies and the heterogeneity of the HBM and hazard data used. The lessons learnt will be applied in PARC projects to be expected to start in 2026. In addition, the Real-life mixtures project tested several statistical methods to study the link between exposure and health effects

Key Words

Mixture risk assessment (MRA), cumulative risk assessment groups (CAG), co-exposure, real-life mixtures, pesticides, metals, PFAS, human biomonitoring (HBM), risk drivers.

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Acronyms

ADI: Acceptable Daily Intake
AO/ AOP: Adverse Outcome/ AO Pathway
BKMR: Bayesian Kernel Machine Regression
BMA: Bayesian Model Averaging
BMDL: Benchmark Dose Lower Level
BMI: Body Mass Index
CAG NAM: Cumulative Assessment Group - Nervous system/Acute/Motor division effects
CAG NAN: Cumulative Assessment Group - Nervous system/Acute/Neurochemical effects
CKD: Chronic Kidney Disease
CI: Confidence Interval
CRA: Cumulative Risk Assessment
CSS: Chemicals Strategy for Sustainability
DG ENV: Directorate-General for Environment
DG GROW: Directorate-General for Internal Market, Industry, Entrepreneurship and SMEs
DG SANTE: Directorate-General for Health and Food Safety
EC: European Commission
EDI: Estimated Daily Intake
EFSA: European Food Safety Authority
EL: Exposure Level
ENET: Elastic Net Regression
EU: European Union
FAIR: Findable, Accessible, Interoperable, Reusable
FT4: Free tetraiodothyronine
FUE: Urinary Excretion Factor
GB: Governing Board
GDPR: General Data Protection Regulation
HBM: Human Bio-Monitoring
HBM-GV: Human Biomonitoring Guidance Value
HBM-RP: Human Biomonitoring Reference Point
HBM-TV: Human Biomonitoring Toxicological Values
HDMA: High Dimensional Mediation Analysis
HQ: Hazard Quotient
HS: Horseshoe Regression
IQ: Intelligence Quotient
ISCED: International Standard Classification of Education
LB: Lower Bound
LOD: Limit of Detection
LOQ: Limit of Quantification
MAF: Mixture Assessment Factor

MB: Middle Bound
MCMC: Markov Chain Monte Carlo
MCRA: Monte Carlo Risk Assessment
MOE: Margin of Exposure
MOET: Total Margin of Exposure
mpMLR: Multiple-pollutant Multiple Linear Regression Models
MRA: Mixture Risk Assessment
mRPI: modified Reference Point Index
NAM: New Approach Methodology
NOAEL: Non -Adverse Effect Level
OCP: Organochlorine Pesticide
OR: Odds Ratio
OS-OA: One Substance-One Assessment
PARC: Partnership for the Assessment of Risks from Chemicals
PBK: Physiologically Based Kinetic
PC: Principal Component
PCA: Principal Component Analysis
PCB: Polychlorinated Biphenyl
PD: Parkinson Disease
PEQ: PFOA Equivalent
PFAS: per- and polyfluoroalkyl substances
PIP: Posterior Inclusion Probability
POD: Point of Departure
P6.2.3: PARC Project 6.2.3
QGcomp: Quantile G-computation
QIVIVE: Quantitative in vitro-in vivo Extrapolation
RCR: Risk Characterisation Ratio
RF: Random Forest
RP: Reference Point
RPF: Relative Potency Factor
RPQ: Reference Point Quotient
SG: Specific Gravity
SHBG: Sex Hormone-Binding Globulin
SNMU: Sparse Non-Negative Matrix Underapproximation
spMLR: Single-pollutant Multiple Linear Regression Models
SVHC: Substances of Very High Concern
TAs: total Arsenic
TDAR: T-Cell Dependent Antibody Response
TIDAR: T-Cell-Independent Antibody Response
TPO: Thyroid Peroxidase

TRA: Toxicologically Relevant Arsenic
TSH: Thyroid-Stimulating Hormone
TT3: Total Triiodothyronine
TT4: Total Thyroxine
TWI: Tolerable Weekly Intake
T6.2: PARC Task 6.2
UB: Upper Bound
UF: Uncertainty Factor
VIMP: Variable Importance
WP6: PARC Work Package 6
WQS: Weighted Quantile Sum

Glossary

See additional deliverable AD6.5 “Development of the strategy from mixture risk assessment using HBM data and its application to prioritised mixtures” (PARC, 2023)

1. Introduction

Exposure to multiple chemicals in real-life situations and the combined effects that this may have generates growing concern among scientists and society. Currently in Europe, Agencies and National Authorities perform risk assessment of chemicals on a substance-to-substance basis, or in a few cases for a limited number of substances from the same chemical family that are expected to have a common effect. People are exposed to a wide range of chemicals through a variety of sources and there is growing consensus that the effect of chemical mixtures needs to be considered in the risk assessment process. In response to this, the Chemicals Strategy for Sustainability (CSS) proposed several ways in which chemical mixtures can be better addressed in the future (European Commission, 2020a). The aim of the Project (P) 6.2.3 Real-life mixtures within the European Union (EU) funded Partnership for the Assessment of Risks from Chemicals (PARC) project, is to develop practical tools, data, methods, and a general strategy to enable an effective mixture risk assessment (MRA) to address policy questions. Currently, the Plant Protection Products Regulation is the most advanced regulatory sector with regards to MRA and implementing the European Food Safety Authority (EFSA) MRA guidance documents (EFSA, More, et al., 2019; EFSA et al., 2021). Therefore, this will be considered in the developments within PARC. Additionally, PARC will align with work done in other EU funded projects which collected human biomonitoring (HBM) data. Notably, HBM data plays a fundamental role in the developments of this Real-life mixtures project.

The PARC project Real-life mixtures is focused on MRA based on HBM data. It continues the lessons learned from the European Human Biomonitoring Initiative (HBM4EU) project and the priorities set by DG SANTE, EFSA and other stakeholders. This project is considered to be complementary to EFSA's methods. It addresses the gap of missing aggregated exposure data as outlined in 2019 in the SWD (250) document supporting the CSS and it is recommended in the EFSA and OECD guidance (EFSA et al., 2021; European Commission, 2020b; OECD, 2018). This project runs in close interaction with PARC Task (T) 4.1.4 which aims to collect HBM data. Furthermore, the integrated model and data platform in PARC T8.3 and Work Package (WP) 7 are cooperating on making tools and data accessible for future risk assessors and risk managers.

The additional deliverable AD6.5 "Development of the strategy from MRA using HBM data and its application to prioritised mixtures" summarised the context of the Real-life mixtures project, its objectives, the proposed MRA strategy, the HBM data that were intended to be harmonised and analysed, and the selection and the application of the strategy to the prioritised case studies (PARC, 2023). This deliverable describes the implementation of the proposed strategy across Europe with all partners involved including EU agencies (Sections 3 and 4) and discusses the obtained results (Sections 5-10) and their relevance for risk management of chemical mixtures (Section 11). Besides, the engagement of European Agencies in this project and dissemination activities, webinars and discussions with regulators are described in Section 2.

PARC is not mandated to perform formal risk assessment. The CSS and other documents identified the need for filling data and knowledge gaps related to MRA since it is not addressed sufficiently in regulation. Consequently, the project Real-life mixtures was highly prioritised by regulators (PARC Governing Board members) and PARC stakeholders to fill these data gaps.

2. Regulatory relevance and working towards the One Substance One Assessment requirements

2.1. Requirements by legislation

Regulatory relevance is of utmost importance for the PARC projects in general and for the PARC project Real-life mixtures in particular. This priority is set by the PARC Governing Board (GB) and used in the evaluation of PARC projects and deliverables. Therefore, there is a need to understand the priorities set by the PARC GB, the regulations addressing MRA and the work done in parallel by the European Agencies. MRA is described in a few EU directives, but in many other regulations a mandate for MRA is lacking. The gap in regulations and knowledge how exposure via multiple exposure routes was identified at the start of the project.

At the start the PARC partnership in 2022, regulators and stakeholders were asked to identify chemical groups and/or endpoints that should be considered in PARC projects. The priorities were set on endpoints for example neurotoxicological or immunological relevant endpoints. Furthermore, priorities were set on groups of chemicals for example per- and polyfluoroalkyl substances (PFAS) and metals. Priorities were set for optimising innovative methods for future MRA research. The case studies described in this deliverable were selected based on the agreed PARC priority process.

Currently only the pesticide legislation (Regulation (EC) No 396, 2005; Regulation (EC) No 1107, 2009) specifically include requirements for MRA. DG SANTE and the regulators of the EU Member States agreed on an action plan on how to implement MRA methods in the coming years (EFSA, 2021). In 2024, the methods were further developed. In a co-creation process, EFSA, RIVM and WR-BIOM developed standard regulatory actions in the Monte Carlo Risk Assessment (MCRA) software, which is a key-model in the PARC model network (EFSA, 2024a; Engel et al., 2024; van Klaveren et al., 2024). A regulatory action is offering the Regulatory mandated organisations to perform a MRA, which is called cumulative risk assessment (CRA) in the Pesticide Regulation. Furthermore, training has been provided to regulatory end-users. Basically, there are two assessments to be done, one retrospective CRA for the pesticides that has been grouped into a Cumulative Assessment Group (CAG) and that are already in use in practice and one for new pesticides for which the mixture risk needs to be assessed before it can be registered according to the Pesticide Regulation.

For other legislation, the methods and tools for MRA are less advanced as they are for pesticides. However, DG SANTE has mandated EFSA to work on many scientific opinions describing how MRA can be performed. In areas other than pesticides, assessments were conducted by EFSA on groups of substances with similar adverse outcomes. The findings from these assessments are presented in various EFSA opinions, which are published for public awareness (EFSA CONTAM Panel et al., 2017; EFSA CEP Panel et al., 2019; EFSA CONTAM Panel et al., 2020; Battilani et al., 2020). Based on the need to provide guidance on grouping applicable in MRA, EFSA was mandated to provide guidance. In 2021, EFSA published the Guidance Document on Scientific criteria for grouping chemicals into assessment groups for human risk assessment of combined exposure to multiple chemicals (EFSA et al., 2021). From then onwards, EFSA intend to apply these criteria in all EFSA domains.

Scientific criteria such as hazard-driven criteria can be used to group these chemicals into assessment groups. In this guidance document, a framework is proposed to apply hazard-driven criteria for grouping of chemicals into assessment groups using mechanistic information on toxicity as the gold standard where available (i.e., common mode of action or common key-events in adverse outcome pathways). However, when such mechanistic data is not available, grouping may be performed using a common adverse outcome e.g., chemicals affecting an organ. Prioritisation methods include combined risk-based approaches, risk-based approaches for single chemicals and exposure-driven approaches. This prioritisation approach enables EFSA to identify substances that may be of potential concern.

The CSS has emphasised the need to address risk assessment of both intentional and unintentional mixtures in European regulations. The CSS highlights the possible need for a Mixture Assessment Factor (MAF), allows for dedicated approaches for MRA when data is sufficiently available and to strengthen the use of collecting, harmonising and using HBM data for understanding the exposure data collection.

Regarding human risk assessment of chemical mixtures, many activities have been finalised since the European Commission has published SWD 250 and the CSS (European Commission, 2020b, 2020a), and new initiatives to proceed on the recommendation of the strategy were initiated. Before starting the PARC project Real-life mixtures, these achievements and initiatives were overviewed.

Although the conclusions from the workshops aiming to discuss the MAF under REACH have not been published yet, many European projects have recently published work on MRA. The MAF is still under discussion in the context of the revision of the REACH regulation (personnel communication from CARACAL meeting 3-4 April 2025 (ref to be added once available)).

On 7th December 2023 the European Commission published draft regulation proposals. Two proposals are extremely relevant for the PARC project Real-life mixtures (European Commission, 2023e). The first proposal is the COM(2023) 779 proposal for establishing a common data platform on chemicals, laying down rules to ensure that

the data contained in it are findable, accessible, interoperable and reusable and establishing a monitoring and outlook framework for chemicals (SWD(2023) 855) (European Commission, 2023a, 2023d). The proposed common data platform on chemicals would include data on physicochemical properties, hazards, uses, exposure safety, risks, occurrence, emissions, environmental sustainability of chemical substances and on ongoing regulatory processes. It would build on existing tools, notably IPCHEM, the Information Platform for Chemical Monitoring. The proposal also aims to establish systematic collection of human biomonitoring data generated in the EU. The proposed regulation would empower the European Chemicals Agency to generate data when needed.

The second proposal is the COM(2023) 783 proposal for amending Regulations (EC) No 178/2002, (EC) No 401/2009, (EU) 2017/745 and (EU) 2019/1021 of the European Parliament and of the Council as regards the re-attribution of scientific and technical tasks and improving cooperation among EU agencies in the area of chemicals (SWD(2023) 850) (European Commission, 2023c, 2023b).

The one substance one assessment (OSOA) requirements should lead to: increased efficiency and predictability, enhanced consistency of assessments and their outcomes, carried out on the same dataset, improved robustness of the assessments, involvement of the right expertise at the right place at the right time, provision of tailored assessments under specific legislations/ uses if relevant, optimal use of resources (European Commission, 2023e). All these aspects are relevant for the PARC T6.2 “Integrative exposure and risk assessment”, including the PARC project Real-life mixtures.

During its April 2025 plenary session, the European Parliament adopted its position on a package of three proposals aimed at making safety assessments of chemicals faster, simpler and more transparent by optimizing the work of EU agencies involved and creating a common data platform on chemicals (Vivienne Halleux & Members’ Research Service, 2025).

Moving towards implementation of the OSOA requirements, actions at multiple levels are required, including:

- Upfront close coordination of assessments across different DGs, scientific committees and agencies at European level; this also includes stronger coordination of Member State’s regulatory initiatives;
- Data exchange across committees and agencies;
- Application of a one hazard assessment;
- Centralising exposure assessment tools and methodology on a common platform;
- Securing tailored risk management;
- Increasing transparency on the decisions and processes. These different elements are further detailed below.

When discussing the OSOA package, the European Parliament and some Member States asked for a systematic collection of HBM data generated within the EU. This is required to bridge the knowledge and data gaps observed in exposure assessment of aggregated/ multiple sources and routes. Furthermore, it will inform policy makers about the levels of chemicals found in humans (e.g., in blood, urine or breast milk) that has been identified as needed for future risk assessment. The proposal is to continue the PARC aligned study beyond 2029 (end of PARC).

According to the OSOA regulatory requirements HBM data generated in the EU funded projects must be stored in the common data platform on chemicals under the responsibility of the European Environmental Agency (EEA). The methods and models as developed in this PARC project Real-life mixtures and implemented into the PARC model platform could be linked to the common data platform on chemicals. The Monte Carlo Risk Assessment (MCRA) software, which is a key-model in the PARC model network, is already linked to the IPCHEM database (Bopp et al., 2018), and an application programming interface between MCRA and the EFSA data framework will be established (Engel et al., 2024; van Klaveren et al., 2024). In the next steps of the PARC project Real-life mixtures work will be initiated, and discussion will be held with the European Agencies on how to link the concepts and models to the common data platform.

2.2. Working together with European Agencies

The PARC Real-life mixtures project and more globally the T6.2 Integrative exposure and risk assessment are working with EU agencies to propose data, methods and tools to perform risk assessment in considering multiple chemicals and sources (and routes) of exposure.

According to its strategy, EFSA aims that by 2030 the Agency and its partners will be ready for the routine implementation of human health risk assessment to multiple chemicals across its domain of activity. Currently, such data, tools and methods have not been fully developed and additional work is needed before a routine implementation can be achieved. Therefore, EFSA called for a Roadmap for action on the Risk Assessment of Combined Exposure to Multiple Chemicals (RACEMiC). This Roadmap was published in October 2022 (de Jong et al., 2022). In addition to MRA, EFSA aims to efficiently conduct aggregate exposure assessments for chemicals using both exposure models and HBM data by 2030. To achieve EFSA's goal, a roadmap for action for advancing aggregate exposure in the EU was developed (ExpoAdvance). This roadmap was created by performing a series of engagement and data collection activities to map the currently available methods, data, and tools for assessing aggregate exposure of chemicals, against the needs and priorities of EFSA (Lamon et al., 2024).

This allowed for the prioritisation of an aggregate exposure assessment framework, identification of data and knowledge gaps in our current capabilities, and identification of the challenges and blockers that would hinder efforts to fill the gaps. The roadmap identifies interdependent working areas where additional research and development are required to achieve EFSA's goals. It also proposes future collaboration opportunities and recommends several project proposals to meet EFSA's goals. Eight proposal projects supported by Strengths, Weaknesses, Opportunities et Threats (SWOT) analysis are presented for EFSA's consideration. The project proposals inform high-level recommendations for multi-annual and multi-partner projects. Recommendations to improve stakeholder engagement and communication of EFSA's work on aggregate exposure assessments were gathered by surveying stakeholders on specific actions to improve EFSA's communication on aggregate exposure, including webinars, virtual training, social media channels, and newsletters.

There is continuous communication between EFSA and the Real-life mixtures project team. A training was organised for EFSA staff on the 19th March 2024. The training was well-attended by 25 EFSA key-experts in risk assessment. The case study leaders and some key-experts of the Real-life mixtures project trained EFSA staff on how HBM data are organised in Europe and an overview of HBM data availability and accessibility for MRA in the MCRA software was provided. The relevant regulatory requirements set in the European Privacy Regulations were explained. The PARC case study leaders explained the concepts, organisation of hazard, exposure and kinetic data for the case studies of PFAS affecting the immune system and pesticides affecting the nervous system. EFSA staff were offered hands-on training on mixtures of pesticides and PFAS using HBM data sets. They learnt how to generate relevant output based on example data using the MCRA software (e.g., run risk actions and use the outputs (i.e. pie charts and plots) to understand the risk of combined exposure via multiple exposure routes (aggregated exposure) using HBM data.

Furthermore, EFSA and PARC organised a joint meeting at the EFSA Advisory Forum meeting (De Brouwere et al., 2024; Dujardin, 2025; EFSA, 2024b). The EFSA Advisory Board is composed of directors of National Food Safety Authorities. The key take-home messages from the session reinforced the essential role of aggregated exposure, kinetic models and HBM data in achieving robust risk assessments and effective risk management. Participants agreed that closer collaboration among Member States, EFSA, other EU Agencies and PARC is vital for its success, and they recognised the growing need for comprehensive consumer product databases and a better communication of findings at the national level. Continuous advancements in data collection and methodologies remain crucial for addressing challenges and refining the accuracy of aggregated exposure assessments. Moving forward, the focus will remain on developing a comprehensive EU framework for aggregated exposure assessment that unites expertise and resources from multiple sources, ensuring a more complete understanding of chemical exposures and fostering effective management strategies across the EU. PARC will continue to work on aggregated exposure, MRA using HBM data and model integration until 2029. Training will be given, and PARC training might also be offered to the Food Safety Authorities of the Member States.

In the context of OS-OA regulatory requirements and the EFSA's strategy on ExpoAdvance and RACEMiC, EFSA organised a meeting with other European Agencies and PARC T6.2 integrative exposure and risk assessment.

The PARC T6.2 leaders and ECHA started to discuss the need for webinars and an increasing interest of ECHA staff to be informed about the PARC integrative exposure and risk assessment projects. A number of meetings were organised by ECHA with the T6.2 leaders. The use of HBM data and non-dietary sources of exposure for plasticisers were highlighted as topics for co-operation in the short-term. In the longer-term, webinars or training will be

organised on how PARC integrative exposure and risk assessment concepts and/or models can be optimised for ECHA.

The PARC project Real-life mixtures and the EEA's role in the Common Data Platform were presented in the International workshop HBM data from regional level to international level, which was organised by the Ministry of Flanders and PARC on the 5th of June 2024 (*Conference, 2025*; Jacob van Klaveren, 2025; Magnus Løfstedt, 2025). The EEA and the PARC project Real-life mixtures started the discussion on how to align the MRA innovation as described in this deliverable to their duties in the OS-OA regulations. Furthermore, the importance of exposure of outdoor/ emission data and how this may contribute to Real-life mixtures concern is now under discussion.

As a follow-up of the international workshop HBM data from regional to international level, discussions were initiated by the EEA and on their responsibilities of data collection related to emission data. RIVM started to explore how these data are being organised and whether these data can be used for risk assessment or not. This requires kinetic models and access to emission data.

The EEA is also responsible for developing indicators linked to the CSS. This work was developed in the HBM4EU project in closed cooperation with the EC-Joint Research Centre.. The CSS includes indicators to track progress towards this goal, particularly focusing on chemical mixtures, which are complex and can present unique risks. These indicators help to identify and manage the impacts of chemical mixtures on human health and the environment. The basis for the mixture indicator was described in Socianu et al., (2022). The EU has developed a dashboard of 25 quantitative indicators and 22 signals to assess the drivers and impacts of chemical pollution, including those related to chemical mixtures. Meetings were organised aiming to discuss the strengths and weakness of the chemical mixture indicator concluding that a signal is not equal to a scientific based mixture risk assessment.

2.3. Interactions with PARC Governing Board, DG SANTE, DG GROW, DG ENV and DG-JRC

On the 15th 2023 November, the PARC GB discussed the need for a webinar on integrative risk assessment. ANSES, VITO, WUR and RIVM are co-organising a series of trainings (e.g., for the European Agencies and stakeholders) and webinars for risk managers on integrative exposure and risk assessment and real-life mixtures using HBM data. Some members of the PARC GB are participating in committees of DG SANTE or DG ENV. To offer equal information to all members of these committees, all members of these committees were invited. The webinar was attended by:

- GB members;
- Colleagues of GB members participating in DG SANTE and Standing Committee of Plant, Animal, Food and Feed;
- Colleagues of GB members participating in DG SANTE Working Group on Industrial and Environmental Contaminants;
- Colleagues of GB members participating in DG ENV CARACAL (an informal Commission expert group which advises the European Commission and ECHA on questions related to the REACH and CLP Regulations).

The content of the webinar focused on:

- Using HBM data for MRA;
- Understanding the sources and routes of exposure and potential options for risk reduction;
- Integrative risk models for MRA compliant with FAIR (Findable, Accessible, Interoperable and Reusable) principles.

The webinar was attended more than 100 regulators from 22 Member States. It was positively evaluated at the PARC GB Meeting on the 16th of May 2024. The impact of the webinar was highlighted in the PARC indicator framework as being a success story on how to engage regulators at the national and the international level in PARC projects.

From the PARC GB webinar onwards, follow-up discussions were organised. A meeting was held on the 18th of January 2025 at the premises of DG SANTE. The PARC Real-life mixtures case study leaders presented their work. We discussed the state of play related to the regulatory implementation of MRA and how regulators should be engaged in the next years. DG GROW, DG ENV and DG-JRC showed interest in follow-up activities to discuss the regulatory perspective of integrative risk assessment results for regulatory interventions (see Section 11).

3. Strategy of PARC P6.2.3 Real-life mixtures

The proposed strategy to perform MRA from HBM data, is built upon the approach and guidance developed in the literature and by EU agencies (EFSA et al., 2021; European Commission, 2020b; OECD, 2018). This strategy is organised in three steps: 1) prioritisation of chemical mixtures of concern for human health and the associated effects; 2) collection and organisation of exposure, hazard and toxicokinetic data required to perform MRA; 3) development and application of methods and tools for MRA of the prioritised substances and their associated adverse health effects. More details, on the strategy and methods were presented in the additional deliverable AD6.5 (PARC, 2023).

From PARC priorities and discussions with EU agencies and the European Commission, pesticides, metals and PFAS were prioritised among other chemicals, as well as four specific health effects of concern which were neurotoxicity, nephrotoxicity, immunotoxicity and developmental neurotoxicity. Overall, four case studies were addressed in the Real-life mixtures project and are presented in this deliverable: 1) Pesticides – neurotoxicity – Section 5; 2) Metals – nephrotoxicity – Section **Erreur ! Source du renvoi introuvable.**; 3) PFAS – immunotoxicity – Section 7; 4) Metals – developmental neurotoxicity – Section 8. More realistic mixtures crossing regulatory silos are being identified in HBM datasets using statistical analysis – Section 9. To note, mycotoxins were not finally considered due to the lack of available HBM data at this stage of the PARC project.

The selected case studies reflect the heterogeneity of available hazard and exposure data from HBM datasets that can be used in MRA. Considering the additivity hypothesis (i.e., adding up individual effects to predict the likely impact of the overall mixture), one main challenge to perform the risk assessment is to collect appropriate hazard values associated with the selected common adverse health effect of the mixture components. As the use of HBM data to perform risk assessments is recent, the establishment of internal toxicological values was necessary to compare to measured internal concentrations (e.g., HBM data) as part of human health risk assessments. Human Biomonitoring-Guidance Values (HBM-GV) are internal toxicological values based on the critical (i.e., most sensitive) effect that corresponds to an internal exposure level at or below which there is no expected health risk. HBM-GVs are derived by experts on the basis of toxicological and epidemiological data according to scientifically accepted derivation schemes (HBM4EU, 2017, 2021). Regarding epidemiological data, internal health-based guidance values (i.e., HBM-GVs) can be derived based on associations between internal chemical concentrations and an observed effect. As the critical effect upon which the HBM-GVs are based for each component of the MRA can differ from the selected common effect of the MRA in question, Human Biomonitoring – Threshold Values (HBM-TV), which are internal toxicological values based on the common mixture effect, can be established. Similar methods to those used to derive HBM-GVs are used, considering the common effect rather than the critical effect of the substance in question (see Metals case studies, Section **Erreur ! Source du renvoi introuvable.** and 8). One common starting point for establishing HBM-G/TVs can be the use of already established external health-based guidance values (e.g., ADIs or TDIs). Considering toxicokinetic data, such values can be converted to internal health-based guidance values (i.e., HBM-GVs) using forward dosimetry. In this project, to compensate for the absence of internal toxicological data which hinder the derivation of internal toxicological values (HBM-GVs, HBM-TVs), HBM exposure concentrations can be converted to external exposure concentrations (e.g., estimated daily intakes (EDI)) using toxicokinetic data and compared with an external reference point (e.g., NOAEL) (see Pesticides case study, Section 5). More detailed about the hazard data strategy can be found in the section 5.1.2 of the AD6.3 (PARC, 2023).

5When an HBM-GV is available from one mixture component for the common effect and internal Relative Potency Factors (RPFs) are available for the other components, risk assessment can be performed using the RPF approach (PFAS case study, Section 7). When no RPFs are available, the ratio between the HBM exposure and the HBM-G/TV can be calculated for each mixture component (Metals case studies, Sections **Erreur ! Source du renvoi introuvable.** and 8). Kinetics data and models related to the prioritised mixtures were collected to be applied directly or to determine key parameters such as urinary excretion fractions (FUE) to convert internal to external exposure concentrations (see Pesticides case study, Section 5).

In addition, the Real-life mixtures project tested several statistical methods to study the link between exposure and health effects. A selection of statistical methods was applied to several HBM studies with sufficient information on biomarkers of exposure and biomarkers of effects. A summary of this work is described in Section 10.

Applying the MRA strategy necessitated work related to data collection, organisation and harmonisation, the implementation of the strategy, hazard data (e.g., HBM-GV/TV) alignment, statistical analysis, kinetic modelling in the MCRA software to be able to perform risk assessment (Section 4). MCRA is a web-based platform addressing the full chain for aggregated hazard, exposure and risk assessment for health effects in the general population and in specific populations (e.g., workers). It is part of the PARC model network which is developed in T8.3. Work was also performed in close cooperation with WP7 (linked to the project and via data champions) and WP4 on exposure data generation.

4. Prerequisites for implementing the strategy

4.1. Human biomonitoring data organisation

As part of the PARC Real-life mixtures project, HBM datasets for general and occupational populations across Europe were organised so that they could be used for MRA. Firstly, an inventory of the available HBM datasets across Europe was created. The inventory comprised datasets that were accessible through partners of this PARC project. A summary of this inventory can be found in the additional deliverable AD6.5 (PARC, 2023).

To securely analyse the individual HBM data in MCRA, data controller-data processor agreements were organised between the RIVM (data processor) and the collaborating institutes (data controllers) according to the General Data Protection Regulation (GDPR).

The next step involved the harmonisation and validation of the HBM datasets in line with the FAIR principles which promote the findability, accessibility, interoperability and reusability of data. Data harmonisation and validation were done according to the approach and criteria outlined in HBM4EU and maintained by VITO (<https://hbm.vito.be/tools/data-harmonization>). The three key steps in the process of data harmonisation and validation were as follows:

Step 1: Fill the biomarker list

Step 2: Fill the empty data template using instructions of the codebook

Step 3: Data validation

Overall, the harmonisation of individual HBM and accompanying data is essential to make them FAIR and it also improves the comparability between HBM studies. By harmonising the data, it was also made more compatible with the MCRA toolbox.

In total, 31 datasets from 14 countries were harmonised and analysed using the MCRA toolbox. An overview of the HBM datasets, including study name, is displayed in Figure 1. Additional details of the HBM datasets are displayed in Table 1. The datasets are quite diverse and heterogeneous regarding the sample size (ranging from n=30 to n=5654), sampling time periods (ranging from 2000 to 2023), biomarkers of exposure measured, and analytical methods used. Various chemicals were measured in different target populations including children, adolescents, pregnant women, adults and workers. These groups represent a diverse cross-section of European society and provide valuable insights into the exposure of different populations.

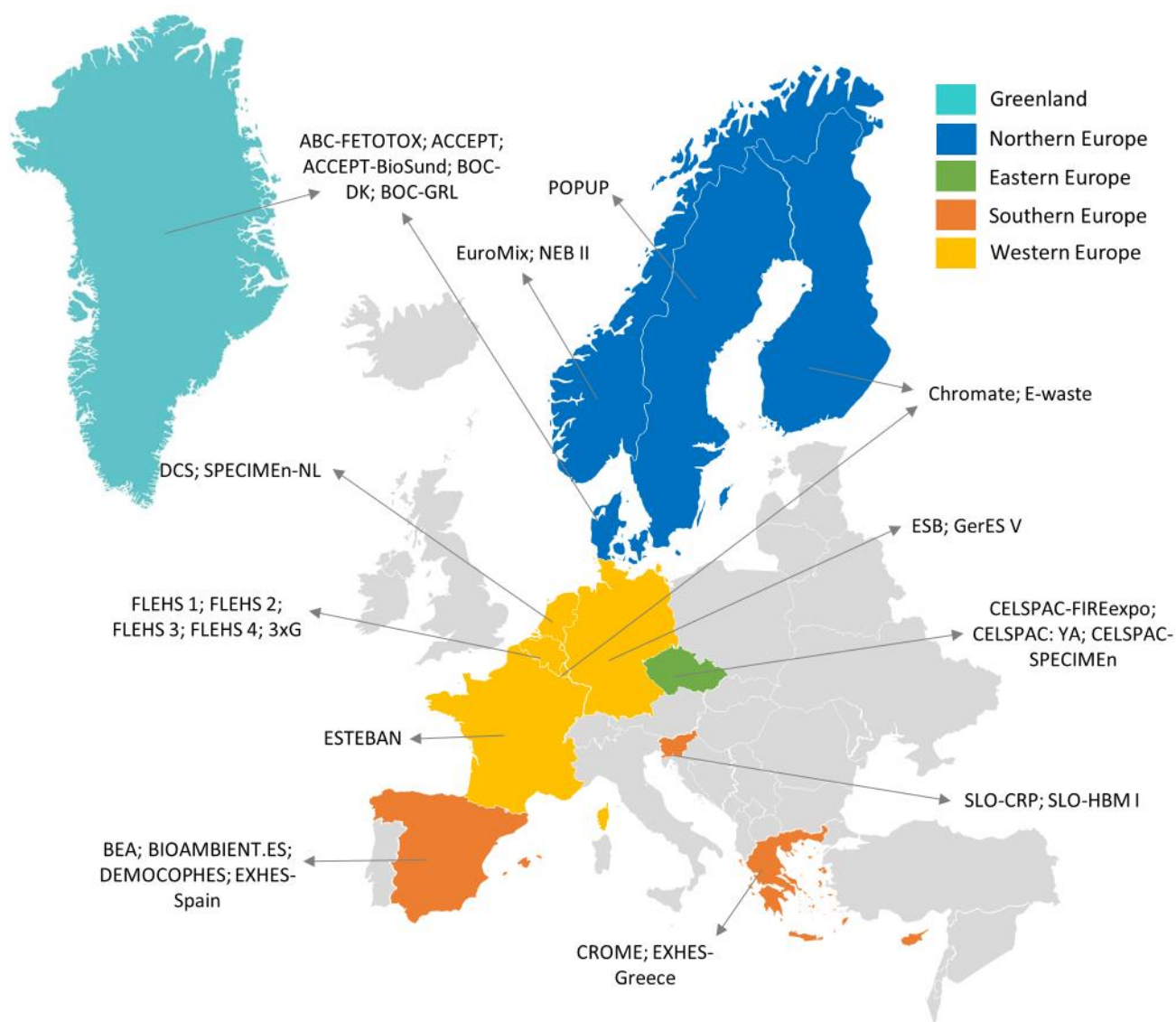


Figure 1. An overview of the HBM datasets organised and analysed with the MCRA toolbox within the PARC project Real-life mixtures. Per country, the study name(s) are provided.

Table 1. Additional details of the HBM datasets organised and analysed with the MCRA toolbox within the PARC project Real-life mixtures.

Dataset acronym	Full study name	Country/Region	Study design	Sampling period	Study population	Age range	Sample size (number of individuals)
ABC-FETOTOX	Aarhus Birth Cohort	DK	Cross sectional	2008-2014	Pregnant women	18-45	1533
ACCEPT	Greenlandic Birth Cohort	DK/GRL	Cross sectional	2010-2015	Pregnant women	18-48	580
ACCEPT-BioSund	Follow-up of the ACCEPT Greenlandic Birth Cohort	GRL	Longitudinal	2013-2020	Children Adults	3-6 20-55	97 166
BEA	Biomonitorización En Adolescents	ES	Cross sectional	2017-2018	Adolescents	13-17	300
BIOAMBIENT.ES	Biomonitoring Study of Environmental Contaminants	ES	Cross sectional	2009-2010	Adults	18-66	1892
BOC-DK	Breast cancer risk in Denmark	DK	Longitudinal	1996-2002	Pregnant women	20-42	412
BOC-GRL	Breast cancer risk in Greenland	DK/GRL	Cross sectional	2000-2014	Adults	18-81	245

CELSPAC: YA	Central European Longitudinal Studies of Parents and Children - Young Adults	CZ	Longitudinal	2019	Adults	20-37	300
CELSPAC-FIREexpo	Central European Longitudinal Studies of Parents and Children - firefighter exposition	CZ	Cross sectional	2019-2020	Workers	18-35	165
CELSPAC-SPECIMEn	Central European Longitudinal Studies of Parents and Children - Survey on Pesticide Mixtures in Europe	CZ	Cross sectional	2020	Children Adults	5-12 31-54	110 110
Chromate	Occupational Chromate Study of the HBM4EU project	FI, LU	Cross sectional	2018-2020	Workers Controls	20-68 23-63	399 203
CROME	Cross-Mediterranean Environment and Health Network	EL	Cross sectional	2020-2021	Children Adolescents Adults	6-11 12-18 19-69	161 150 258
DEMOCOPHES	Consortium to Perform Human biomonitoring on a European Scale	ES	Cross sectional	2011-2012	Children Adults	6-11 26-48	120 120
DCS	Doetinchem Cohort Study	NL	Cross sectional	2018-2019	Adults	52-91	336
ESB	Environmental Specimen Bank	DE	Cross sectional	2015-2022	Adults	20-29	2589
ESTEBAN	Etude de Santé sur l'Environnement, la Biosurveillance, l'Activité physique et la Nutrition	FR	Cross sectional	2014-2016	Children Adolescents Adults	6-11 12-17 18-75	526 552 2478
E-waste	Occupational E-waste Study of the HBM4EU project	FI, LU	Cross-sectional	2020-2022	Workers Controls	18-64 21-60	300 150
EXHES-Greece	Exposure and Health Examination Survey Greece	EL	Cross sectional	2020-2023	Adults	23-52	99
EXHES-Spain	Exposure and Health Examination Survey Spain	ES	Cross sectional	2018	Pregnant women	18-45	60
EuroMix	European Test and Risk Assessment Strategies for Mixtures	NO	Cross sectional	2016-2017	Adults	24-72	144
FLEHS 1	Flemish Environmental and Health Studies 1	BE	Cross sectional	2002-2005	Children Adolescents Adults	0 13-16 49-65	2392 1679 1583
FLEHS 2	Flemish Environmental and Health Studies 2	BE	Cross sectional	2008-2011	Children Adolescents-ref Adolescents-Genk ¹ Adolescents-Menen ² Pregnant women Adults	0 13-16 13-19 13-17 18-42 21-40	255 210 197 199 255 204
FLEHS 3	Flemish Environmental and Health Studies 3	BE	Cross sectional	2013-2014	Children Adolescents-ref Adolescents-Genk Pregnant women Adults	0 13-16 13-16 18-44 49-65	281 208 200 281 209
FLEHS 4	Flemish Environmental and Health Studies 4	BE	Cross sectional	2017-2018	Adolescents	13-16	428
GerES V	German Environmental Survey for Children and Adolescents	DE	Cross sectional	2014-2017	Children Adolescents	3-11 12-18	956 1327
NEB II	Norwegian Environmental Biobank II	NO	Longitudinal	2016-2017	Children	7-14	668
POPUP	Persistent Organic Pollutants in Uppsala Primiparas	SE	Longitudinal	2015-2019	Women Children	22-48 4-12	203 59
SLO-CRP	Exposure of children and adolescents to selected chemicals through their habitat environment; Slovenia	SL	Cross sectional	2018	Children Adolescents	7-10 12-15	149 97
SLO-HBM I	Slovenian HBM programme	SL	Cross sectional	2008-2014	Adults	18-49	617
SPECIMEn-NL	Survey on Pesticide Mixtures in Europe - Netherlands	NL	Cross sectional	2020	Children Adults	6-11 30-56	102 105
3xG	Health Municipalities Births	BE	Longitudinal	2011-2021	Children Pregnant women	0-8 23-44	513 301

¹ Adolescents from the region Genk in Flanders, Belgium.

² Adolescents from the region Menen in Flanders, Belgium.

4.2. Hazard data organisation

As part of the Real-life mixture project, relevant hazard data required in the MRA for the case studies were collected and/or derived such as HBM-GVs, HBM-TVs and NOAELs (see Sections 5.2.1, 6.2.1, 7.2.1 and 8.2.1). In order to use this data, the values were retrieved and placed in MCRA format Excel files for each case study. The format allows inclusion of information about the variability of the values, when possible (e.g., age-dependant HBM-TV in the PFAS case study) and the uncertainty (e.g., distribution of HBM-TVs in the Metals case studies).

4.3. Development of the MCRA software for PARC P6.2.3

To operationalise the HBM-based MRA strategy outlined in Section 3, it was essential to develop a computational workflow that effectively integrates multiple models and data sources. These integrations are being implemented through the PARC model network – a digital ecosystem that is developed in PARC T8.3 to provide European researchers, regulators, and other stakeholders access to a comprehensive suite of harmonised workflows for integrative chemical risk assessment.

Since the case studies varied greatly in terms of target populations, substances, and health effects, and because the heterogeneity in available hazard and exposure data, tailored variants of the generic MRA strategy were applied in each case study. For example, the PFAS case study uniquely incorporated internal RPFs, while the pesticide case study was the only one to translate observed HBM concentrations into external exposure estimates using kinetic models. The metals case studies required calculation of toxicologically relevant arsenic (TRA) levels from studies that reported either total arsenic (TAs) or individual arsenic species, necessitating appropriate conversions.

To accommodate this heterogeneity, the workflow was designed and implemented in the PARC model network to be both broadly applicable and sufficiently flexible, ensuring consistent, reproducible assessments across all case studies, and making it applicable beyond the case studies presented here.

4.3.1. Main requirements

Early in the project, several core requirements for the workflow were defined. The **primary requirement**, already noted, **was that the workflow should be generic and capable of accommodating a wide range of modelling variations based on the availability of data**. Beyond this, the following key requirements were identified:

User friendly and transparent: security restrictions require HBM data controllers to run the workflow themselves, keeping the HBM data in their control, yet still producing harmonised outputs. Accordingly, the workflow must be easy to use, support harmonised analyses at the European level, and ensure full transparency, including publication of the model source code and enabling export of complete, reproducible, assessment files.

Data privacy: since the HBM-based MRA strategy relies on individual-level HBM data, the workflow must be implemented in a GDPR-compliant and secure tool that prevents unauthorised access to raw data. Its output must likewise safeguard privacy by avoiding disclosure of sensitive information, for example suppressing high-percentile exposure estimates that would identify small numbers of individuals.

HBM data: the tool must directly link to the HBM data organisation and adhere to the data standard formats established by PARC (see Section 4.1). To ensure full transparency, the workflow should accept individual-level raw HBM data as its input and carry out subsequent processing steps as part of the workflow, while ensuring data privacy as mentioned above. All subsequent processing steps, such as normalisation, standardisation, censored-value imputation, and missing-value imputation, must follow the procedures defined in the PARC T4.1 statistical analysis plan.

Other data: next to HBM data, the workflow must support a biomarker catalogue (substance list), RPFs, hazard characterisations, biomarker and kinetic conversion factors (see below), and eventually physiologically based kinetic (PBK) model definitions and parametrisations. Wherever possible, these datasets should follow forthcoming harmonised formats established in PARC WP7. To ensure consistent reuse and traceability across case studies, all data must be version-controlled and accessible via a shared repository in the modelling tool.

Connectivity with other workflows: the workflow should link to other components of the PARC model network. Notable links include workflows for aggregate exposure assessments to identify the main sources and routes

driving mixture risk and environmental burden of disease assessment. It already integrates with the chemical co-exposure-based mixture identification workflow implemented in MCRA as described in the PARC additional deliverable AD6.5 (PARC, 2023). Beyond PARC, it relates to EFSA's retrospective and prospective dietary MRA workflows implemented in MCRA.

4.3.2. Key design choices

The MCRA toolbox is a key node of the PARC model network. It implements EFSA's validated methodology for retrospective and prospective dietary MRA. It also supports integrative risk assessment in PARC with several workflows, such as aggregate exposure assessment and environmental burden of disease assessment. It offers a secure, centrally hosted, user interface with robust user- and data-management features, along with open-access model code. Since few public tools support HBM-based risk assessment, MCRA was extended to include the HBM-based MRA workflow.

The MCRA toolbox serves as the main user interface for the workflow. The use of a central platform such as MCRA simplifies the sharing of data, methods and updates, without requiring local software installations, while ensuring harmonised and reproducible analyses. However, GDPR mandates restricted access to individual-level HBM data, which must be uploaded to the secure MCRA toolbox for assessment. To comply, formal data controller-data processor agreements were established between each HBM dataset controller and the RIVM (the MCRA host). These agreements safeguard privacy and give study owners exclusive rights to analyse their own data within the platform (see Section 4.1).

4.3.3. Modular structure

The HBM-based MCRA workflow leverages a fully implemented modular design, built on the MCRA architecture and extensive development work carried out in PARC. Each module, representing one or more modelling tasks, is part of the PARC model network. Workflows or workflow variants combine modules in various configurations to suit data availability and risk assessment strategies. Figure 2 illustrates the modular design implemented for HBM-based MRA, which is a subset of the broader framework of the model network. Embedding within this overarching framework enables interoperability with other workflows now and into the future.

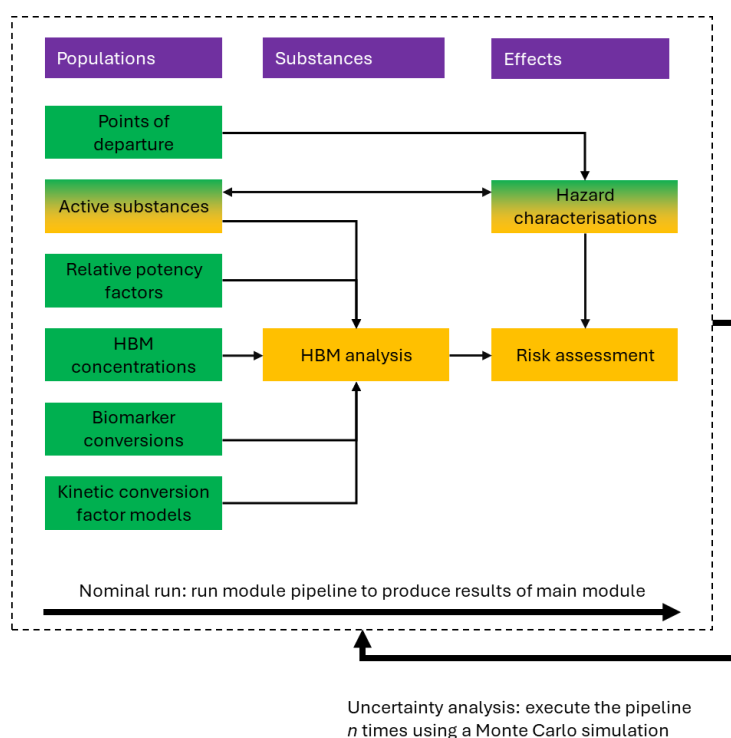


Figure 2. The conceptual modular framework for the HBM-based MRA workflow. It features a network of linked data modules (green boxes) and calculation modules (yellow, representing modelling tools). The pink modules are generic scoping models to define the *population, substances, and health effect in scope*.

Figure 2 illustrates the modules integrated within a two-dimensional Monte Carlo framework designed to:

- a. Capture population variability in the outputs (e.g., the risk distribution)
- b. Quantify the combined uncertainty in the outputs (e.g., a 95% uncertainty interval for a percentile of the risk distribution).

Population variability is derived from individual-level HBM data and from probability distributions to account for variability of a parameter (e.g., biomarker conversions, see below). Overall uncertainty is quantified by bootstrapping the HBM data and by sampling from uncertainty distributions (e.g., for hazard characterisations or kinetic conversion factors, see below).

The following key modules are highlighted in Figure 2:

Risk assessment: The functionality of this core module was expanded in PARC to support assessments based on internal exposure estimates. In MCRA these estimates are either based on modelled exposures (in connection to T6.2.1) or measured exposures (HBM data). In this workflow, it integrates exposure estimates from HBM data in module *HBM analysis* with toxicological values (e.g., No-Observed-Adverse-Effect-Level (NOAEL), HBM-GV or HBM-TV) derived from module *hazard characterisations*. Users can select from a range of risk metrics such as the (*modified*) *Reference Point Index*, the *Margin of Exposure Total* or *Hazard Index* based on a cumulative exposure estimate used in the case studies below (see details about these risk metrics in Sections 5.1, 6.1, 7.1 and 8.1). The main outputs of the module include an estimate of the risk distribution and an analysis of the contributions of risk driving substances to the overall risk. All outputs are presented with uncertainty intervals.

Hazard characterisations: This module was extended to support both internal and external hazard characterisations using harmonised data formats. Hazard characterisations may depend on individual properties like age and gender. In addition, uncertainty within each subgroup can be specified as a distribution. As shown in Figure 2 *hazard characterisations* can inform the *active substances* module about the substances in scope. For example, in the metals case study. Users indicate that the assessment is based on TRA rather than TAs (see Section **Erreur ! Source du renvoi introuvable.**). In the pesticide case study, the *active substances* module directly identifies the substances in scope, with the CAG defined as data in this module (see Section 5.) Hazard characterisations for these substances are then derived from *points of departures* data, following the same process used in MCRA for dietary MRA using EFSA's methodology.

HBM concentrations and HBM analysis: For transparency, the workflow uses raw individual-level HBM data without prior transformations. As detailed in Section 4.1, PARC harmonises HBM data into a standard format. The *HBM concentrations* module lets users to upload their data, select individuals and biological matrices, and convert the data into internal MCRA formats (supporting PARC HBM codebook versions 2.0–2.3). The HBM analysis module contains processed biomarker concentrations following WP4's statistical analysis plan¹ (unpublished internal document), with imputation of censored and missing values as outlined in AD6.5 (PARC, 2023). Blood biomarker concentrations can be standardised for lipid content via outcomes of gravimetric analysis, enzymatic summation, or a derived total lipid value (Bernert et al., 2007). Urine concentrations can be normalised based on specific gravity, with missing values estimated from creatinine levels using different models. Alternatively, urine concentrations can be standardised by creatine concentration. Finally, various biomarker conversions and kinetic conversions can be applied (see below).

Biomarker conversions: Occasionally, the biomarker of interest is not measured. Biomarker conversions transform biomarker values within a biological matrix into new parameters. The output of such a conversion can be any linear combination of observed biomarker values. Examples include summing different arsenic species to obtain TRA or deriving TRA from TAs. For each biomarker, conversion specifications are provided as data in the *biomarker conversions* module, and users can enable these conversions as an option in the *HBM analysis* module. Conversion factors may depend on individual properties like age and gender. In addition, variability between factors within each subgroup can be specified as a distribution.

¹ https://agenceanses.sharepoint.com/:w:/r/sites/PARC-ANSES/_layouts/15/Doc.aspx?sourcedoc=%7BD9082890-23F8-4BD8-A954-D13168089462%7D&file=PARC_R4.1.4_Statistical%20Analysis%20Plan_VERSION1_20231130.docx&action=default&mobileredirect=true

Kinetic conversion factors: Kinetic conversions use a toxicokinetic model to transform measured biomarkers into estimates of either biomarker concentrations in alternative biological matrices or external exposures to the corresponding parent compound (reverse dosimetry). Specified as an option in *HBM analysis*, the current workflow supports low-tier kinetic conversions using pre-defined conversion factors. Future developments aim to integrate higher-tier conversions employing PBK models, in accordance with the PARC FAIR PBK standard (PARC, 2023). Similar to biomarker conversions, case-specific kinetic conversion factors are provided via a dedicated data format, allowing for any linear combination of the measured biomarkers. In the pesticides case study, reverse dosimetry is conducted using conversion factors defined at the subgroup level, including uncertainty distributions.

Relative potency factors: The workflow supports estimation of cumulative exposures from HBM biomarker values by applying internal RPF values. These values are provided as data in the relative potency factors module, and users can provide multiple sets of values to capture uncertainty.

4.3.4. User interface and example

The workflow is accessible through the MCRA web platform at <https://mcra.rivm.nl>. Figure 3 displays the main user interface, which allows users to choose the exposure type (*acute* or *chronic*), the target level (in this workflow, an *internal concentration*), and the exposure calculation method (in this workflow, using *human biomonitoring concentrations*). The risk metric is then determined by specifying the risk characterisation ratio (either *exposure/hazard* or *hazard/exposure*) and selecting the cumulation setting (either the *sum of risk characterisation ratios* or *RPF weighted*). In this example, using an “exposure/hazard” ratio together with the “sum of risk characterisation ratios” option defines the (modified) reference point index (see Section **Erreur ! Source du renvoi introuvable.**). The example shown in Figure 3 illustrates an artificial assessment for a mixture of four metals following the guidance that was developed for the Metals – nephrotoxicity case study. Additional examples of the user interface are shown in AD8.4 and D8.6.

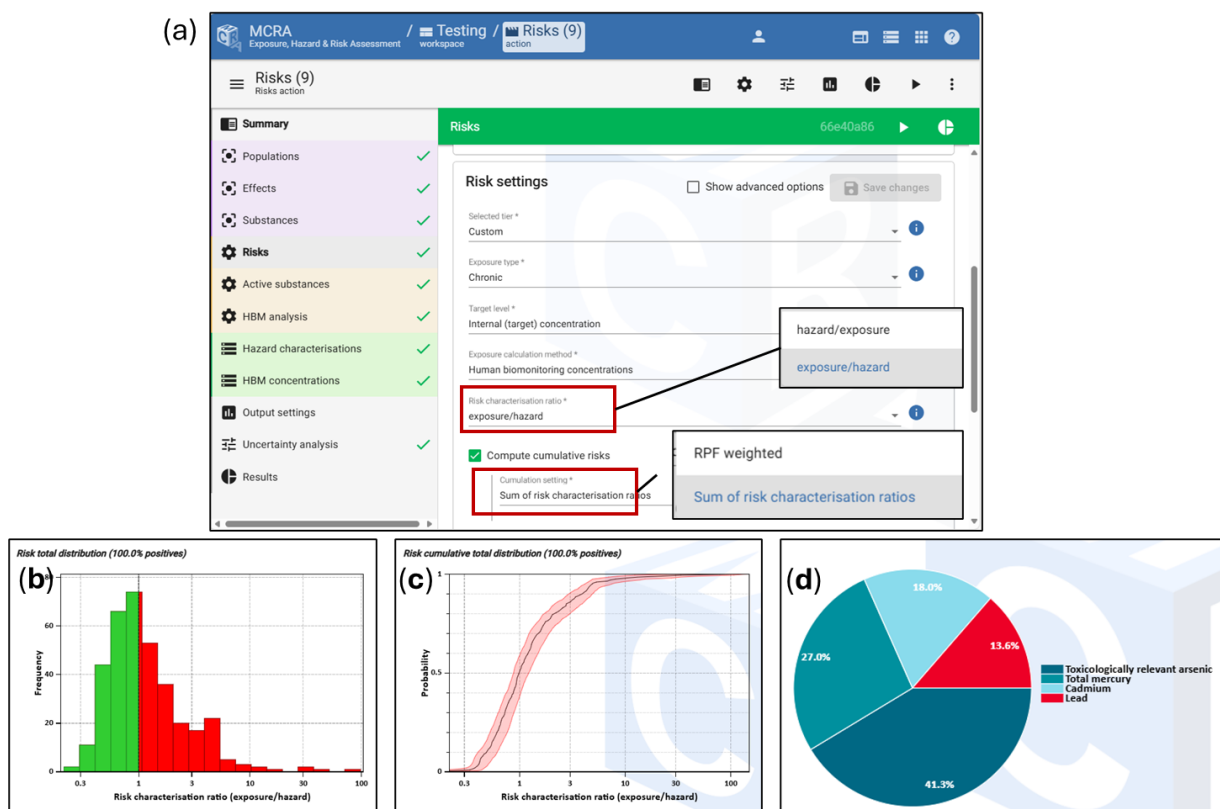


Figure 3. (a) Main user interface of the MCRA workflow for HBM-based MRA, along with examples of outputs (panels b – d) from an artificial assessment for a mixture of four metals and nephrotoxicity. Panel (b) displays a histogram of the risk distribution reflecting the variability observed in the nominal run in Figure 3. Panel (c) presents the risk as a cumulative distribution with an uncertainty interval. Panel (d) presents the average contribution of each substance to the risk for the individuals “at risk” (i.e., exceeding a defined threshold).

4.3.5. Validation

As described in AD8.4 and D8.6, automated unit tests and regression tests were implemented to ensure consistency in the output of the workflow across new releases of MCRA. In addition, the variants of the workflow supporting the PFAS, metals and pesticides case studies were externally validated against an independent implementation. Finally, as part of ongoing acceptance testing the workflow is continuously refined based on feedback from users.

4.4. Training and guidance documents

To ensure harmonised application of the methodological approaches, detailed guidelines were drafted, and training sessions were provided to partners within the PARC Real-life mixtures project. The training sessions were delivered face-to-face and online in October 2023. Guidance documents adapted to each case study were created to guide data controllers' step by step through the methodology, indicating the parameters to be entered for each scenario. The guidance documents include explanations of how to use the MCRA toolbox and screenshots of how to apply each step of the MRA strategy. The guidance documents, training materials and training recordings are available on the P6.2.3 SharePoint.

5. Mixture risk assessment: Pesticides - neurotoxicity

5.1. Mixture risk assessment strategy

Human health risk assessment from exposure to pesticides is traditionally conducted based on external dietary exposure data, taking into account food consumption data, and pesticide residues in the different commodities. The estimated amount of each pesticide consumed via food is compared to the respective Acute Reference Dose (ARfD) as derived from the toxicological data of the chemical or, when dealing with cumulative risk assessment, to the NOAEL for the common effect. The aim of this study was to develop an MRA approach for pesticides associated with a specific target organ i.e., the nervous system, triggering the specific neurotoxic effects. The novelty of the approach is that the exposure estimation is based on HBM data. The detected levels of pesticides and their metabolites could be directly compared to the respective internal thresholds. However, only a few internal thresholds (HBM-GVs) were available for the identified metabolites. Thus, the approach is based on reverse dosimetry applied to urinary concentrations of exposure biomarkers and comparison of the obtained estimated external levels (as an Estimated Daily Intake (EDI)) with NOAELs for the specific effect, as proposed in EFSA acute cumulative dietary exposure assessments. In this case study, we used CAGs as defined by EFSA associated with two acute effects: acetylcholinesterase inhibition (CAG NAN) (including 47 active substances) and functional alteration of motor division (CAG NAM) (including 100 active substances) (EFSA, Crivellente, et al., 2019). More information about the approach can be found in section 9.1 of the additional deliverable AD6.5.5 (PARC, 2023). This approach was applied to different age groups and different populations, i.e., children, adolescents and adults using European HBM datasets.

The equation used to convert the exposure biomarkers as detected in urine to external exposure was the following, as adapted from Šulc et al. (2022). Urine is a non-invasive method that reflects recent exposure to many pesticides and their metabolites. Most insecticides have short half-lives and are rapidly metabolized and excreted in urine (Yusa et al., 2015).

The Equation a considers one urinary metabolite concentration to obtain the external dose of one active substance.

Equation a

$$EDI_s = \frac{HBM_{Exp0_m} \times potentialpresence_{sm} \times CE \times \left[\frac{MW_s}{MW_m} \right]}{BW \times FUE_m}$$

The Equation b considers two or three intermediate metabolites. This term “intermediate” refers to metabolites that could be produced during metabolism of the active substances, but they are relatively short lived or could be further metabolised until they reach the ultimate step of transformation. The Equation b was used when dialkylphosphate (DAP) metabolites were measured.

Equation b

$$EDI_s = \frac{HBM_{Expom1} \times potentialpresence_{sm1} + \left[HBM_{Expom2} \times \frac{MW_{m1}}{MW_{m2}} \right] \times potentialpresence_{sm2}}{BW \times [(FUE_1 \times MW_{m1})]} \times CE \times MW_s$$

Where EDI_s is the Estimated Daily Intake of the parent compound s in $\mu\text{g}/\text{kg bw}/\text{d}$, HBM_{Expom} is the concentration of the metabolite in urine in $\mu\text{g}/\text{g}$ of creatinine, $potentialpresence_{sm}$ is the probability of the parent compound to be present as residue in foods considering the associated metabolite (unitless), CE is the anthropometry- and gender-based reference value for daily creatinine excretion in urine derived for children and adults (g of creatinine/day), MW_s and MW_m are the molecular weights of the parent compound and the metabolite(s) respectively ($\text{g}\cdot\text{mol}^{-1}$), BW is the body weight in kilograms, and the FUE is the Urinary Excretion Fraction and it reflects the ratio between the intake amount of active substance of the pesticide and the amount of metabolite excreted in the urine (unitless).

The two equations were combined and applied to datasets where specific and transient metabolite data were available. For risk characterisation, in line with the EFSA's approach, the EDI is compared to the respective NOAEL values for the specific effect and pesticide: the obtained ratio is called margin of exposure (MOE) (Equation c). Applying the dose addition model, the sum of these ratios was used for the estimation of the total margins of exposure (MOET) for the different populations (Equation d and e).

In EFSA assessments, cumulative exposure was expressed as the total margin of exposure (MOET) at the 50th, 90th, 95th, 99th and 99.9th percentile of the exposure distribution. The SC PAFF Committee identified a MOET of 100 at the 99.9th percentile of the exposure distribution as the threshold of regulatory consideration. In this case study due to limited sample size, we expressed the cumulative exposure at the 50th, 90th and 95th percentiles. We used this threshold of 100 reflecting inter-species and interindividual variability to do our risk characterization (EFSA, 2008).

Equation c

$$MOE_s = \frac{NOAEL_s}{EDI_s}$$

Equation d

$$\frac{1}{MOET} = \sum \frac{1}{MOE_s}$$

Equation e

$$MOET = \frac{1}{\sum_s \frac{1}{MOE_s}}$$

5.2. Data

5.2.1. Hazard data

For each individual substance, NOAEL values from animal experimental studies associated with inhibition of acetylcholinesterase (CAG NAN) or functional alteration of motor division (CAG NAM) were extracted from EFSA reports (EFSA, Crivellente, et al., 2019) and were used to calculate MOET. In our case study, considering the availability of the exposure biomarkers, we used up to 27 out of 47 NOAELs from CAG NAN and up to 39 out of 100 NOAELs from CAG NAM.

5.2.2. Human biomonitoring data

Ten HBM datasets from seven countries were harmonised and analysed in the MCRA toolbox for this case study analysis: ESTEBAN (France), SPECIMEn-NL (the Netherlands), CELSPAC-SPECIMEn (Czech Republic), 3xG (Belgium), FLEHS 2 (Belgium), FLEHS 3 (Belgium), FLEHS 4 (Belgium), SLO-CRP (Slovenia) and EXHES-Spain (Spain), and ESB (Germany). An overview of these HBM datasets, including the study name, is displayed in Figure 4. Those datasets contain urinary concentrations of pesticide exposure biomarkers for children, adolescents and

adults, with sampling periods between 2008 and 2021, and sample sizes between 61 and 752. Available biomarkers included organophosphates biomarkers (3,5,6-trichloro-2-pyridinol (TCPy), diethyldithiophosphate (DEDTP), diethylphosphate (DEP), diethylthiophosphate (DETP), dimethyldithiophosphate (DMDTP), dimethylphosphate (DMP), dimethylthiophosphate (DMTP) and 2-diethylamino-6-methylpyrimidin-4-ol (DEAMPY), one N-methyl-carbamates biomarker (Carbofuran phenol), and pyrethroids biomarkers (3-phenoxybenzoic acid (3-PBA), 4-fluoro-3-phenoxybenzoic acid (4-F-3-PBA), cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (cis-DBCA), cis-3-[2-chloro-3,3,3-trifluoroprop-1-enyl]-2,2-dimethylcyclopropanecarboxylic acid (ClF3CA), and 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (cis/trans-DCCA). There was a huge heterogeneity in terms of analysed biomarkers between datasets. The surveys CELSAC_SPECIMEn, SLO-CRP, and Belgium FLEHS 4 Adolescents were the datasets with the lowest number of urinary biomarkers (TCPy, 3-PBA, and cis/trans-DCCA). ESB (Germany), FLEHS 2 Adolescents, FLEHS 3 Adults, FLEHS 2 Adults (Belgium) and ESTEBAN (France) were the surveys with the most biomarkers (DEP, DETP, DEDTP, DMP, DMTP, DMDTP, TCPy, 3-PBA, cis-DBCA, cis/trans-DCCA, 4-F-3-PBA). The MRA was performed depending on the biomarkers available in the dataset for the associated active substances in the CAGs on a case-by-case basis. We did not try to account for missing biomarkers by setting thresholds for biomarkers or active substances. Table 2 summarises the metabolites and active substances considered in the mixture risk assessment. Organophosphates and N-methyl carbamates active substances are present in both CAG-NAN and CAG-NAM.

Left censored data were treated as follows: concentrations at levels below the LOD were replaced with a value of zero, and those below the LOQ were replaced with half the LOQ value.

In EFSA cumulative risk assessment, as agreed between EFSA and the European Commission, a tiered approach is followed. The first-tier calculations (Tier I) use very conservative assumptions for an efficient screening of the exposure with low risk for underestimation, the second-tier assessment (Tier II) includes assumptions that are more refined but still intended to be conservative.

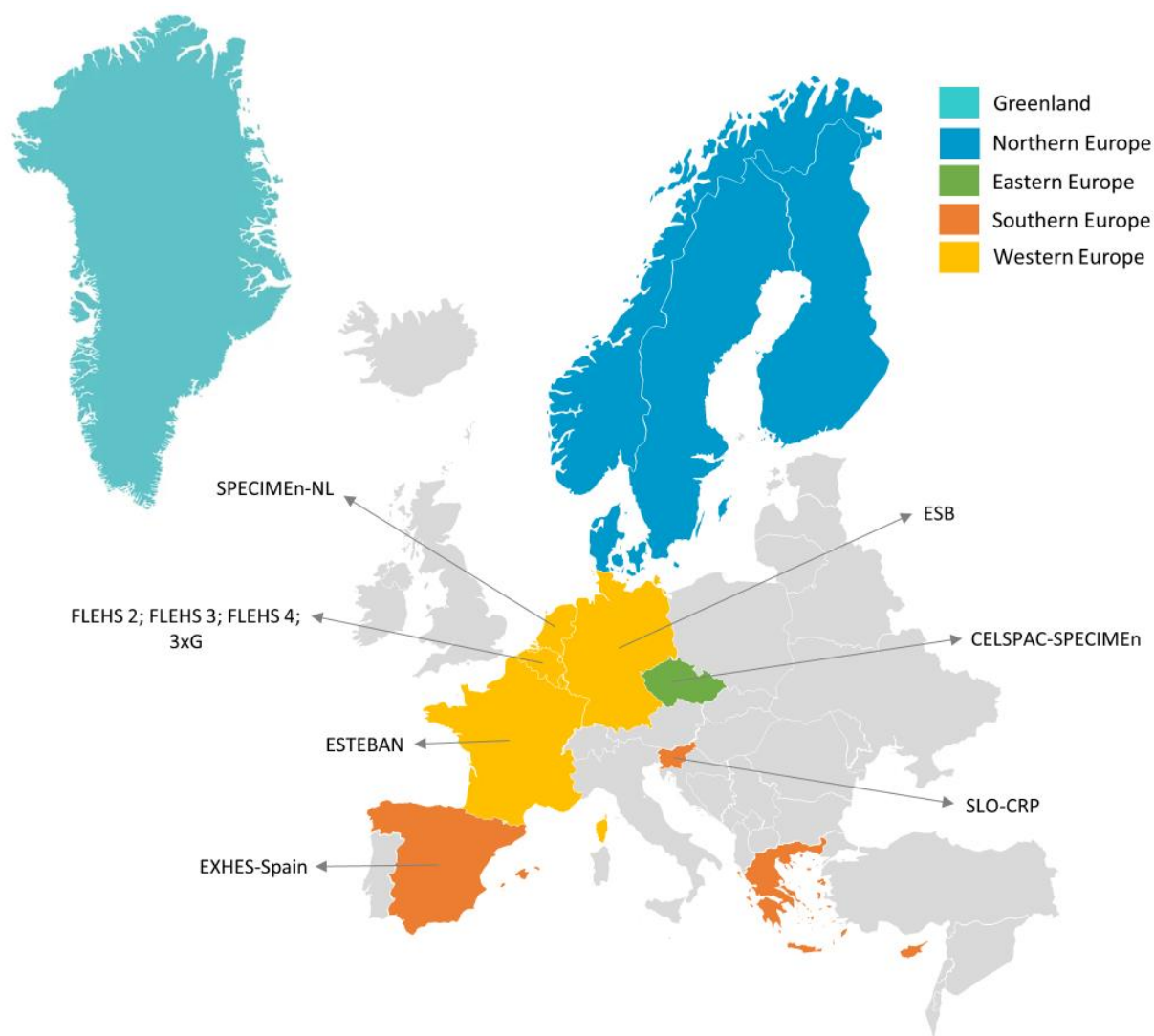


Figure 4. An overview of the HBM datasets applicable for the Pesticides - neurotoxicity case study which were organised and analysed in the MCRA toolbox within the PARC project Real-life mixtures. Per country, the study name(s) are provided.

Table 2 Summary of the considered metabolites for the mixture risk assessment with the associated parent compounds

Family	CAG	Metabolite	Parent compound
Organophosphates	NAN and NAM	TCPy	Chlorpyrifos, Chlorpyrifos-methyl
		DEP	Azinphos-ethyl, Phosalone, Ethion, Triazophos, Diazinon, Parathion, Phoxim, Chlorpfenvinphos
		DETP	Azinphos-ethyl, Phosalone, Ethion, Triazophos, Diazinon, Parathion, Phoxim
		DEDTP	Azinphos-ethyl, Phosalone, Ethion
		DMP	Azinphos-methyl, Malathion, Dimethoate, Methidation, Phosmet, Omethoate, Pirimiphos-methyl, Tolclofos-methyl, Trichlorfon, Dichlorvos, Monocrotophos, Fenthion, Fenitrothion

		DMTP	Azinphos-methyl, Malathion, Dimethoate, Methidation, Phosmet, Omethoate, Pirimiphos-methyl, Tolclofos-methyl, Fenthion, Fenitrothion, Acephate
		DMDTP	Azinphos-methyl, Malathion, Dimethoate, Methidation, Phosmet
N-methyl Carbamates	NAN and NAM	Carbofuranphenol	Carbofuran, Carbosulfan, Benfuracarb
Pyrethroids	NAM	3-PBA	Cypermethrin, Cypermethrin-alpha, Cypermethrin-beta, Cypermethrin-zeta, Esfenvalerate, Fenvalerate, Fluvalinate-tau, Permethrin, Cyhalothrin-lambda
		4-F-3-PBA	Cyfluthrin, Cyfluthrin-beta
		cis/trans-DCCA	Cyfluthrin, Cypermethrin, Permethrin
		cis-DBCA	Deltamethrin
		CIF3CA	Cyhalothrin-lambda

5.3. Mixture risk assessment results

Acute EDIs were obtained for children adolescents and adults, for each active substances considered in the mixture from CAG NAN and CAG NAM. Then the MOET were calculated to characterise the risk associated with acute neurotoxicity. The calculated MOETs for both CAGs could then be compared to the total margin of exposure estimated by EFSA's acute cumulative dietary exposure assessments (EFSA, 2019; van Klaveren et al., 2019).

For CAG NAM, a proportion of children (24%) in one of the seven datasets (ESTEBAN, France) analysed had MOET lower than 100. The risk drivers for that dataset are presented in Figure 5. While for adults, a proportion of individuals (42-93%) in three of the six analysed datasets (ESB, Germany, FLEHS 3, Belgium, and ESTEBAN, France) exceeded the threshold of concern. The risk drivers for two of those three datasets are presented in Figure 6. Regardless of the population, the top three risk drivers were Azinphos-ethyl, Triazophos and Ethion, which were also the main contributors to the risk in EFSA's report. For adolescents, using FLEHS 4 Adolescents survey, all MOETs were higher than 100.

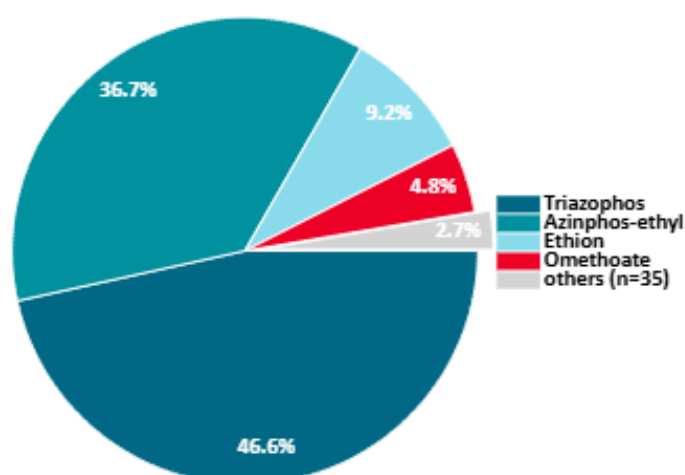


Figure 5. Pesticide contributions to the total margin of exposure (MOET) for CAG NAM in children of the French dataset, ESTEBAN.

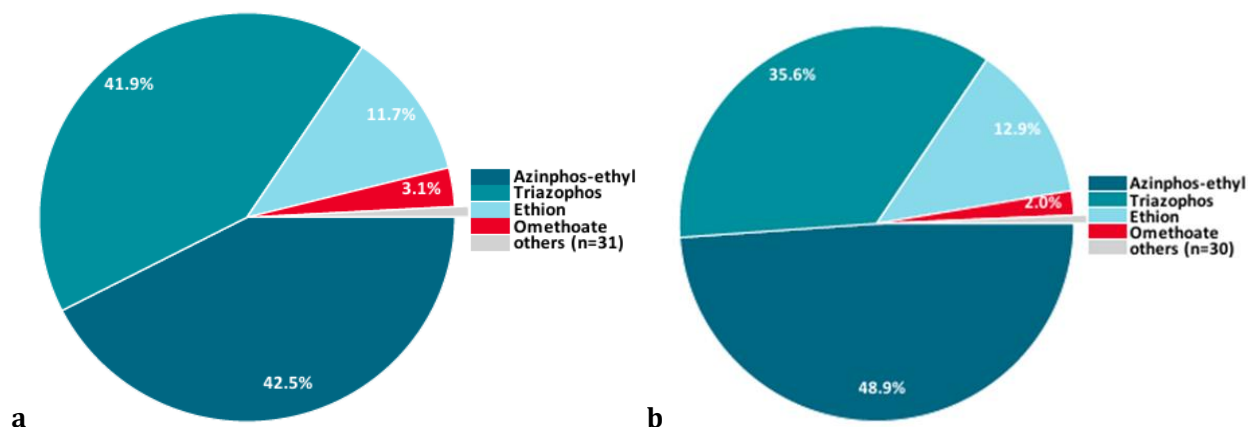


Figure 6. Pesticide contributions to the total margin of exposure (MOET) for CAG NAM in adults of a) the German dataset, ESB, and b) the Belgium dataset FLEHS 3.

For CAG NAN, for children, adolescents and adults all four of the analysed dataset contained a proportion of individuals (3-87%) exceeding the threshold of concern (ESTEBAN, France, FLEHS 2 & 3, Belgium, ESB, Germany). Across the populations, the main risk drivers were Azinphos-ethyl, Triazophos, Omethoate, Carbofuran and Ethion. Similarly, in EFSA's Tier I assessment, the main contributors to the risk were Carbofuran, Triazophos, Chlorpyrifos, Omethoate and Azinphos-ethyl.

Overall, the results from the present case study in several populations and scenarios show a higher level of exposure and risk compared to those estimated by EFSA using dietary exposure data.

5.4. Uncertainties

5.4.1. Uncertainties in hazard data

One of the main challenges, in the absence of internal toxicological threshold values, i.e., HBM-GVs, for specific pesticide biomarkers, is to compare HBM data with the appropriate toxicological threshold values. Thus, to perform MRA, the HBM data were compared to the so far available external toxicological threshold values. For this, the external exposure levels that could correspond to the detected HBM data were estimated through reverse dosimetry.

5.4.2. Uncertainties in exposure assessment

The reverse dosimetry approach included several assumptions, notably the use of default FUE that contributed to the overall uncertainty. Specific urinary excretion factors were only available for a few of the metabolites from the datasets which would have reduced the amount of parent compounds that we could include in this assessment.

Using common pesticide metabolites such as 3-phenoxybenzoic acid (3-PBA), diethyl phosphate (DEP), diethyl thiophosphate (DETP), dimethyl phosphate (DMP), and dimethyl thiophosphate (DMTP) to estimate daily intake of parent compounds is a source of uncertainty as these biomarkers can be formed from the metabolism of several organophosphates. The same applies to the metabolite 3-PBA and its formation from several pyrethroids. Moreover, all active substances present in the CAGs could not be considered in the assessment due to missing exposure biomarkers in the HBM surveys.

In addition, organophosphate active substances and pyrethroids (Demoute, 1989) once applied on crops, can be directly metabolised by plants (in the case of OPs called DAPs) and are dietary consumed through the different food commodities (Tsuchiyama et al., 2022). The ingested metabolites, although toxicologically inactive in most cases, would appear in the HBM measurements and will result in an overestimation of the external exposure (i.e., EDI), and the respective Total Risk. This can explain partly one of the differences in our assessment compared to EFSA results.

Another assumption was the source and routes of exposure to different pesticides. In the current exercise, it was assumed that the detected HBM levels and the respective estimated exposure originate from diet as the only source. However, some pesticides active substances have several uses, such as biocides and veterinary medicines and thus exposure can take place from sources other than food and from other routes than the oral/dietary one.

HBM levels were based on the concentrations detected in one spot urine sample in most datasets from each individual, as well as the corresponding EDI values. This one sample per individual does not reflect the total amount of the metabolite excreted, in addition to inter-individual variability in urinary excretion rate.

For some datasets such as ESB, FLEHS, either the LOD or the LOQ was not available, consequently the proposed left-censored data treatment could have overestimated the estimated exposure levels.

5.5. Future perspectives

The case study presented here is a proof-of-concept for the use of HBM data in retrospective MRA and is not intended to illustrate risks from the substances analysed. In the next steps, this case study will be broadened to consider in the risk assessment other compounds that coincide in the HBM data sets, triggering the same neurotoxic effects. A prioritisation based on the main contributors to the total risk, current approval status of the active substances, their incidence in more recent official residue monitoring programs, and mode of action will be performed in the following months.

In parallel, in collaboration with European Agencies, the need for refinements to the MRA methodology will be explored. For example, regarding the reverse dosimetry approach, the application and impact of additional data and models (e.g., FUEs, PBK models, total diet study data) could be investigated. In particular, refinements could be applied to pyrethroids for which data exists and with the help of PBK modellers from P6.2.2.

6. Mixture risk assessment: Metals - nephrotoxicity

6.1. Mixture risk assessment strategy

Chronic exposure to one specific metal, even at relatively low concentrations, can lead to nephrotoxicity in humans. Additionally, humans often experience combined exposure to mixtures of these metals through multiple routes and sources. Within the framework of the HBM4EU project, an Adverse Outcome Pathway (AOP) was developed for four metals known to induce nephrotoxicity in humans: arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg) (Schillemans et al., 2020). The primary adverse outcome identified was renal dysfunction resulting from proximal tubular damage. However, distinguishing between the effects of these metals on glomerular versus tubular structures proved to be challenging. Consequently, this case study defines the common adverse effect of these metals in humans as nephrotoxicity, with a focus on glomerular and/or tubular damage.

In this case study, HBM data were compared to internal toxicological values called here HBM-TV. The ratios of exposure to hazard of each component can be summed to calculate a modified Reference Point Index (mRPI). The mRPI combines the risk of compounds with a common effect. Based on the common effect a reference point (RP) can be determined for every individual compound. This RP, also known as point of departure (POD), is compared to the respective compound's exposure level (EL), taking different uncertainty factors (UFs) into account. By doing so, a reference point quotient (RPQ) is obtained (Equation f) for each metal. that is comparable to a hazard quotient (HQ) but with the difference that the RPQs are restricted to the same common effect, which may not be the case for the HQs. To combine the exposure and assess the combined risk of the four metals, the default approach of dose addition is assumed. Summing the RPQs of each compound results in the risk metric mRPI (Equation g)

Equation f

$$RPQ_i = \frac{EL_i}{RP_i/UF_i}$$

Equation g

$$mRPI = \sum_{i=1}^n RPQ_i$$

Determining the mRPI can be considered a preliminary (or lower-tier) approach in MRA. To clarify, the HBM-TV for a chemical can be calculated as shown in Equation h, where HBM-RP represents the HBM-Reference Point of the chemical of interest which was established for each compound as the highest concentration of that compound in HBM not triggering the (common) effect (see Section 6.2.1), and UF the Uncertainty Factor associated with this HBM-RP.

Equation h

$$HBM - TV = \frac{HBM - RP}{UF}$$

The objective of this case study is to calculate the mRPI for a mixture of four metals with nephrotoxic effects using HBM data available in Europe and HBM-TVs established in this case study for these metals in blood and/or urine. The mRPI is calculated as follows:

Equation i

$$mRPI = \frac{EL_{TRA}}{HBM TV_{TRA}} + \frac{EL_{Cd}}{HBM TV_{Cd}} + \frac{EL_{Hg}}{HBM TV_{Hg}} + \frac{EL_{Pb}}{HBM TV_{Pb}}$$

Where:

mRPI: modified Reference Point Index

EL: exposure level from HBM data

HBM-TV: Human Biomonitoring-Toxicological Value

The mRPI value is compared to 1 informing that the exposure is higher than the hazard threshold. Thus, a mRPI above 1 means that there may be concern regarding exposure. More information about the MRA strategy of this case study can be found in section 9.2 of the additional deliverable AD6.5 (PARC, 2023).

6.2. Data

6.2.1. Hazard data

HBM-TVs were derived from literature research and are summarised in Table 3.

Table 3. HBM-TV for nephrotoxic effects derived for four metals for general population.

Metal	Matrix	Population for HBM-TV	Effect biomarker	RP (value or range)	UF	HBM-TV (value or range)	References
Cd	Urine	General and workers	β 2-M	2 μ g/g cr. 4 μ g/g cr.	2 3.9 [cumulative risk]	1 μ g/g cr.	(ECHA, 2021; EFSA, 2009; Lamkarkach et al., 2021)
Pb	Whole blood	General	GFR	25-50 μ g/L	[have range for uncertainty]	25-50 μ g/L	(Ekong et al., 2006)
Hg	Urine	General	NAG	6 μ g/g cr.	[value from model]	6 μ g/g cr.	(Apel et al., 2017; HBM4EU, 2022c)
TRA	Urine	General	GFR	10-21 μ g/g cr.	[have range for uncertainty]	10-21 μ g/g cr.	(Hsueh et al., 2009; Lin et al., 2020; Zheng et al., 2015)

β 2-M: β 2-microglobulin, GFR: glomerular filtration rate, NAG N-Acetyl- β 2-D-glucosaminidase, cr.: creatinine

For each of the metals, Table 3 presents the selected values of reference points and uncertainty factors used to derive the HBM-TV. It details the specific biomarker of effect, the biological matrix, and the population from which the reference points were established.

For Cd, urinary Cd reflects long-term, cumulative exposure due to its 10-to-30-year half-life in the kidney and it is a reliable non-invasive biomarker, although adjustments for creatinine or specific gravity are needed to account for diuresis. A toxicological value of 1 µg Cd/g creatinine for urinary cadmium in the general population was established based on meta-analysis and recent evaluations, ensuring protection from cadmium-induced kidney function decline. This value aligns with findings from multiple studies, including EFSA's analysis of epidemiological data, which identified a threshold for renal effects linked to urinary β₂-microglobulin (β₂-M) levels, and the HBM4EU project, which recommended the same threshold for the general population and 2 µg Cd/g creatinine for workers (ECHA, 2021; EFSA, 2009; Lamkarkach et al., 2021). For occupational exposure, studies on battery plant workers were key in determining effect levels, with the lowest LOAEL used to derive conservative protective limits. Considering cadmium's cumulative nature and its risks to older and retired workers, a precautionary approach was adopted, applying an HBM-TV of 1 µg Cd/g creatinine universally across all groups. This decision reflects alignment with key findings and ensures protection for vulnerable populations, including the elderly and workers post-retirement.

Pb is best assessed through whole blood, which reflects chronic exposure due to a steady equilibrium with soft tissues and bones, while urinary Pb is less reliable and reflects recent exposure. For Pb a LOAEL of 50 µg/L blood is used as starting point, based on the review by Ekong et al., (2006). The proposed LOAEL of 50 µg/L for lead nephrotoxicity is suggested as a practical starting point for the assessment of real-life mixtures, with considerations. First, regarding uncertainty and overestimation, the LOAEL is derived using concurrent blood lead levels, which reflect prolonged exposure during a time of declining lead levels. This may slightly overestimate risks. The LOAEL of 50 µg/L is considered a minimal effect level with low concern, and the actual NOAEL (No Observed Adverse Effect Level) is likely close to the LOAEL. Secondly, the standard assessment factor of 3 (LOAEL-to-NOAEL extrapolation) is reduced to 2, based on uncertainties estimated to range from 25 µg/L to 100 µg/L for the general population and workers. For the general population, a NOAEL of ~25 µg/L is suggested as a realistic estimate, balancing between a stringent EFSA value of 15 µg/L and the LOAEL. For workers, reduced intraspecies variability (compared to the general population) supports using 50 µg/L as a reference level for long-term kidney protection, with levels up to 100 µg/L not being an immediate concern. The large available database makes further intraspecies adjustment factors unnecessary. Finally, the rising prevalence of susceptible populations (e.g., those with hypertension, diabetes) is acknowledged, though better diagnostics and treatments may influence prevalence figures. Overall, while a definitive NOAEL for lead-induced nephrotoxicity is difficult to establish, the LOAEL of 50 µg/L is considered a pragmatic and low-concern starting point in risk assessments. A range of 25 µg/L to 50 µg/L is supported for interpreting risk assessment outcomes for the general population and workers, respectively.

Hg exposure depends on its chemical form: inorganic and elemental Hg are assessed using urine as a biomarker, especially for nephrotoxicity, whereas blood and hair are used for methylmercury (MeHg) exposure, with urine often adjusted for dilution variations. Thus, urine is the preferred biological matrix for chronic Hg exposure assessment, as it reflects inorganic Hg levels and body burden. The biomarker N-Acetyl-β₂-D-glucosaminidase (NAG) was deemed suitable for assessing Hg's health effects. The German Commission for Human Biomonitoring established HBM-I and HBM-II values for Hg exposure based on occupational studies. HBM-I represents a no-risk concentration, while HBM-II signifies the threshold for potential adverse health effects, prompting action to reduce exposure. For the general population and workers, the Commission selected 20 µg/g creatinine for HBM-II, while HBM-I was set at 5 µg/g creatinine (7 µg/L) due to no adverse effects being observed below this level. Research noted increased NAG activity in workers exposed to >35 µg/g creatinine Hg. Recent PBK modelling derived a value of 6 µg/g creatinine (or 7 µg/L) in urine, aligning with prior findings (HBM4EU, 2022a). Ultimately, 6 µg/g creatinine was chosen as the toxicological benchmark for assessing inorganic Hg-related nephrotoxicity in risk assessments.

Arsenic (As) exposure is assessed through urine, with focus on toxic-relevant arsenic species (called toxicologically relevant arsenic (TRA) and defined as the sum of arsenite (As(III)), arsenate (As(V)), and their methylation metabolites, monomethylarsonate (MMA) and dimethylarsinate (DMA)) rather than total arsenic (TAs), given dietary influences from organic arsenic sources like seafood. Despite that, TAs is still used usually to assess urinary

As exposure. Guidance values for As exposure are based on health effects like dermal toxicity, vascular issues, cancer, and chronic kidney disease (CKD). EFSA recently updated its threshold for inorganic arsenic (iAs) intake to 0.06 µg/kg bw/day, with benchmark dose lower levels (BMDLs) of 0.12 and 0.06 µg/kg bw/day for CKD (EFSA CONTAM Panel et al., 2024). Studies suggest urinary TRA concentrations of 10–21 µg/g creatinine are associated with CKD risk. This is based on the following studies: Hsueh et al. (2009) observed higher TRA levels (20.74 µg/g creatinine) in Taiwanese CKD patients compared to controls (11.78 µg/g), with estimated Glomerular Filtration Rate (eGFR) reductions beginning at 11.78 µg/g. Zheng et al. (2015) reported a 1.2 hazard ratio for CKD in American Indians within 9.7–15.6 µg/g creatinine. Chen et al. (2011) linked total urinary As (35–75 µg/g creatinine) with kidney damage, which, when adjusted for TRA using European conversion factors (0.2–0.5), equates to 7–37.5 µg/g creatinine. Furthermore, Lin et al. (2020) proposed 1.557 µg/kg bw/day as a threshold for iAs impacting eGFR, which, after applying an uncertainty factor, yields an external toxicological value of 0.493 µg/kg bw/day. This converts to an internal value of 10.2 µg/L in urine, aligning with the 10–21 µg/g creatinine range for CKD risk. These findings highlight consistent evidence for TRA-associated kidney risks and provide a foundation for health-based guidance values in urine.

6.2.2. Human biomonitoring data

Several of the HBM datasets harmonised and analysed into the MCRA toolbox for this PARC project contained metal concentration data. To be suitable for this case study, the HBM datasets were required to have concentration data of at least two metals in the appropriate biological matrix (reflective of chronic exposure) measured in adults to be considered as a mixture. Where HBM datasets include two or more but not all four of the biomarkers of exposure in the target biological matrices, the missing biomarkers are not taken into account in the MRA. The adult population is defined as any individual aged 18 or over. Table 4 **Erreur ! Source du renvoi introuvable.** outlines the different biomarker of exposure-biological matrix pairs selected in this case study. Regarding the biomarkers of exposure, for As, we selected TRA) and TAs in urine. For Pb, concentrations of Pb in whole blood or urine were selected. For Hg, concentrations of total Hg in urine were selected, and finally, for Cd, concentrations in urine were chosen.

Table 4. Biomarker of exposure-biological matrix pairs selected in this case study.

Metal	Biomarker	Biological matrix
Arsenic	TRA or Total As	Urine
Lead	Pb	Whole blood or urine
Mercury	Total Hg	Urine
Cadmium	Cd	Urine

In total, 13 HBM datasets from 10 institutes and 8 countries across Europe were included in this case study (Figure 7). The specific biomarker of exposure-biological matrix pairs measured in the datasets are outlined in Table Table 4. Overall, the datasets were heterogeneous. Across the datasets, individuals from the general population were aged from 18 to 91 years. The sampling period of all datasets ranges from 2004 to 2023. The sample sizes of the studies ranged from 43 to 1689 individuals.

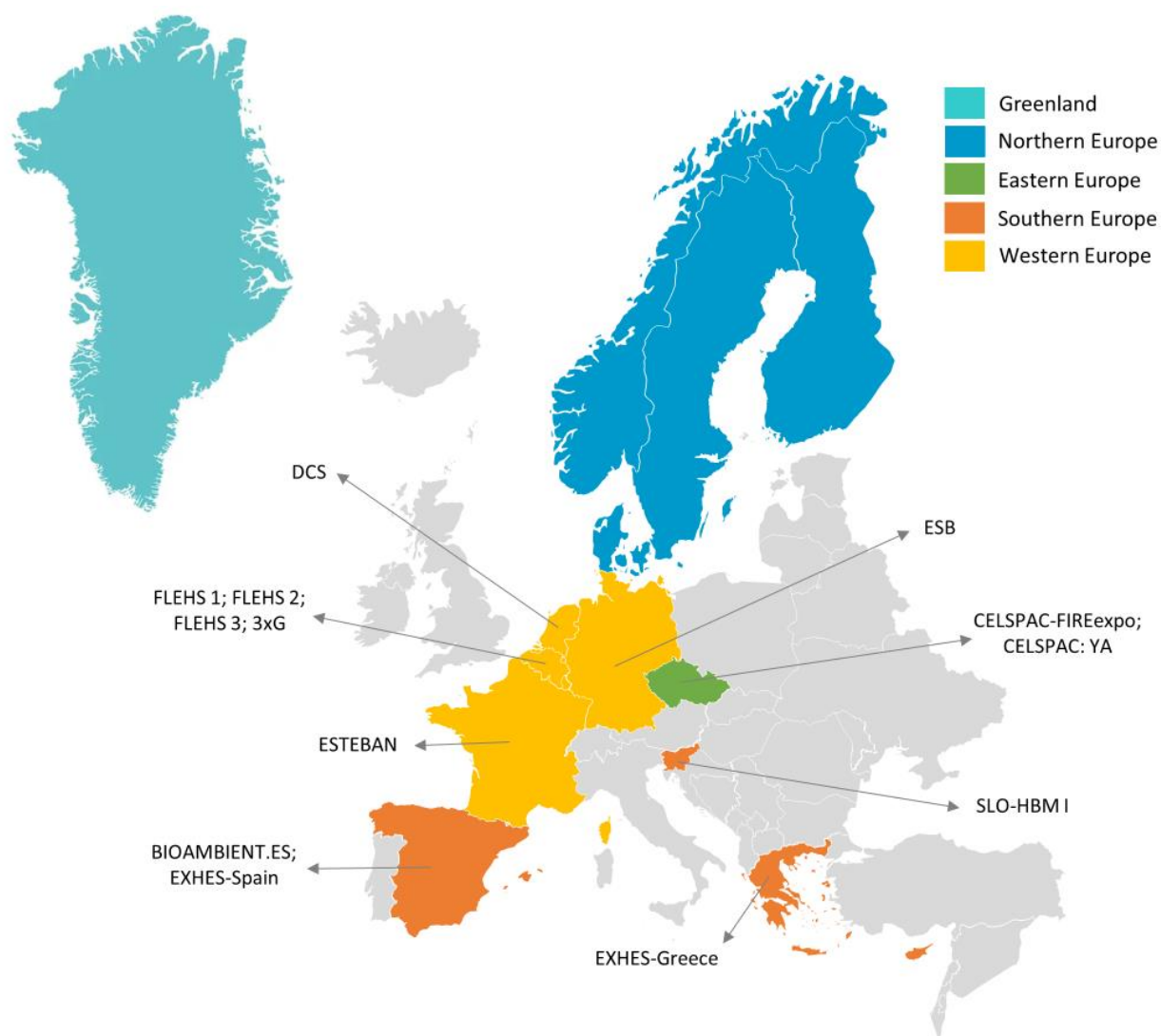


Figure 7. An overview of the HBM datasets applicable for the Heavy metals - nephrotoxicity case study which were organised and analysed in the MCRA toolbox within the PARC project Real-life mixtures. Per country, the study name(s) are provided.

Table 5. Specific biomarker of exposure-biological matrix pairs measured in the HBM datasets included in the heavy metals - nephrotoxicity case study. The data represent adults from the general population.

Dataset acronym	Country	Biomarker of exposure	Biological matrix
BIOAMBIENT	ES	Cd Pb Total Hg	morning urine whole blood morning urine
CELSPAC: YA	CZ	Total As Cd Pb Total Hg	spot urine spot urine spot urine spot urine
CELSPAC-FIREexpo	CZ	Total As Cd Pb Total Hg	morning & spot urine morning & spot urine morning & spot urine morning & spot urine
DCS	NL	As(III), As(V), MMA, DMA, Total As Cd Pb Total Hg	morning urine morning urine whole blood morning urine
ESB	DE	Total As Cd Pb	24-h urine 24-h urine whole blood

		Total Hg	24-h urine
ESTEBAN	FR	TRA, Total As Cd Pb Total Hg	morning urine morning urine whole blood morning urine
EXHES-Greece	EL	Total As Cd Pb Total Hg	spot urine spot urine spot urine spot urine
EXHES-Spain	ES	Total As Cd Pb Total Hg	spot urine spot urine spot urine spot urine
FLEHS 1	BE	Cd Pb	spot urine whole blood
FLEHS 2	BE	TRA, Total As Cd	morning urine morning urine
FLEHS 3	BE	Total As Cd Total Hg	spot urine spot urine spot urine
SLO-HBM 1	SL	Total As Cd Pb Total Hg	spot urine spot urine whole blood & spot urine spot urine
3xG	BE	TRA, As(III), As(V), MMA, DMA, Total As Cd Pb	morning urine morning urine morning urine

To account for variations in diuresis which can be caused by (de)hydration, time of day and interindividual differences, the urinary biomarkers were adjusted for creatinine concentration.

Some biomarker conversions were needed to fit the biomarker-biological matrix pairs for which the HBM-TV were derived. Regarding As, the HBM-TV was derived for TRA in urine. In some HBM surveys, the individual As species were measured. To calculate TRA, the concentrations of As(III), As(V), MMA and DMA were summed on a molar basis. In addition, in many of the HBM surveys, TAs was measured. A conversion factor was derived to convert TAs concentrations to TRA concentrations using the ratio of TRA/TAs from seven HBM surveys which analysed both TRA and TAs. A beta distribution was fitted to the TRA/TAs ratio (mean: 0.435, variance: 0.027) and used to generate a distribution of conversion factor values. For Pb, the HBM-TV was derived for Pb in whole blood. Since some HBM surveys measured Pb in urine, a conversion factor was used to convert concentrations of Pb in urine to concentrations of Pb in whole blood in order to take into account as many biomarkers of exposure and HBM datasets as possible. However, the added uncertainty of this conversion is acknowledged. Based on a PBK model developed by Gastellu et al., (2025) conversion factors for women and men, respectively, were derived (33.57 and 27.05).

Regarding the treatment of left censored data (i.e., concentrations below the LOD and LOQ values), three scenarios were applied. For the lower bound (LB) scenario, censored values were substituted by 0. A middle bound (MB) scenario was used to randomly attribute values between 0 and the LOD or LOQ value according to a log-normal distribution. For the upper bound (UB) scenario, values below the LOD or LOQ were respectively substituted by the LOD or LOQ value.

6.3. Mixture risk assessment results

The MRA strategy applied to heavy metals and nephrotoxicity was performed on 13 HBM datasets across Europe. Considering all scenarios (LB, MB, UB), the percentage of individuals with an mRPI exceeding the threshold of 1, ranges from 21% to 98% for the datasets when four of the targeted metals were included.

These findings indicate potential concern for health effects on the kidney due to current exposure. To note, some HBM datasets have a much higher percentage of individuals exceeding the threshold compared to others. However, the very high percentage of exceedance (up to 98% of the population) seems inconsistent with the known prevalence of kidney damage and therefore it might indicate that this lower tier MRA approach needs refinements.

It is also important to note that the datasets are heterogenous (e.g., different heavy metals measured, sample sizes, sampling periods, etc.), therefore, making it difficult to compare results between countries.

The risk drivers of the mixtures are presented in Figure 8, where the percentage contribution of the individual metals to the overall mRPI for the middle-bound scenario is shown. The lower and upper bound scenario results are not presented, but very similar results were obtained. Where measured, Pb was most often found to be the risk driver of the mixture (8/11 datasets). In the other 3 datasets, TRA was found to be the risk driver for one dataset and Cd was the risk driver for the other two datasets.

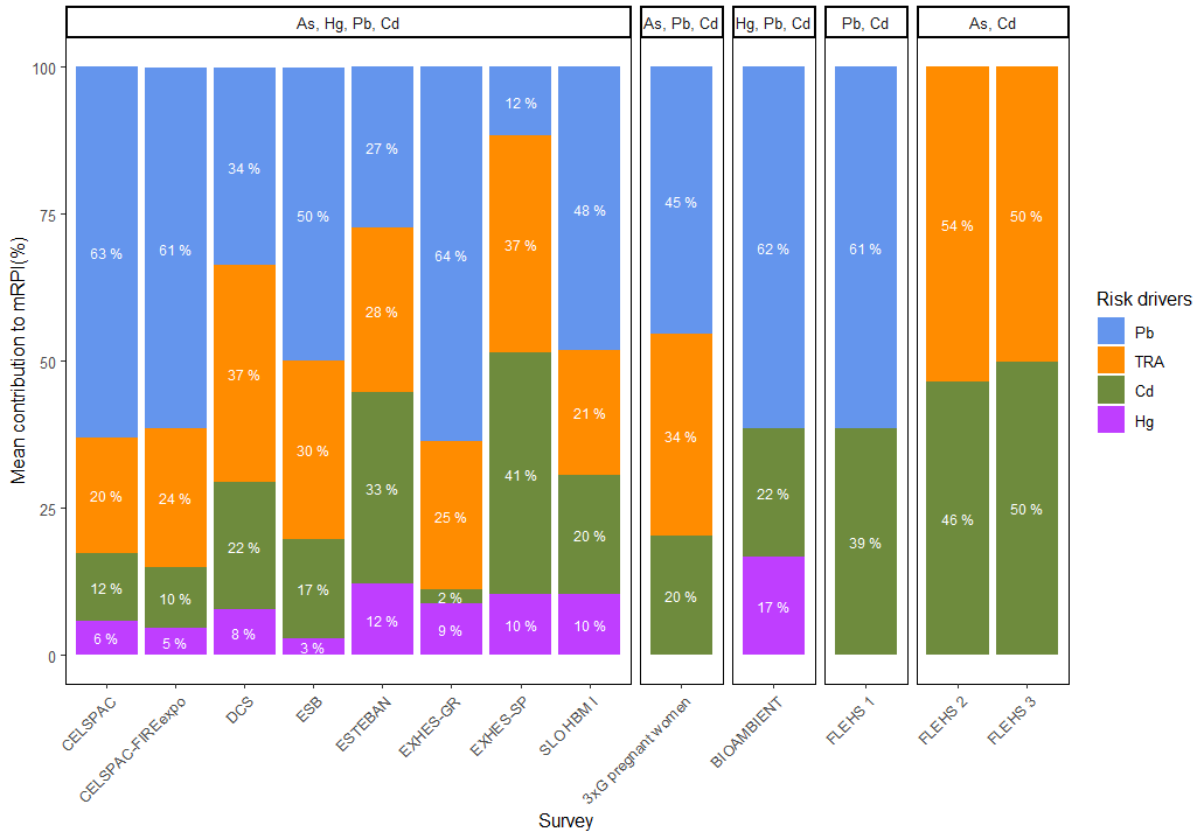


Figure 8. Individual metal contributions to the overall mRPI in adults (middle bound scenario).

6.4. Uncertainties

6.4.1. Uncertainties in mixture risk assessment methodology

The mRPI strategy for the MRA is based on the summation of all individual metal risk ratios. However, each of the metals have other biomarkers for the nephrotoxic effect, adding uncertainty. This lower tier approach can be refined by using the relative potencies of the hazards of the mixture components (see future perspectives, Section 6.5). Furthermore, interactions and/or synergy of the kidney effect of metals are not taken into account.

6.4.2. Uncertainties in hazard data

Regarding the hazard, an HBM-GV with nephrotoxicity as an endpoint has been established by EFSA only for Cd, therefore we derived toxicological values (HBM-TVs) to apply the mRPI approach. The assessment of nephrotoxicity from Cd, Pb, Hg, and As can have uncertainties stemming from study design limitations, biomarker reliability, inter-individual variability, exposure history, and methodological constraints. Many studies rely on cross-sectional designs, which inherently limit causal inference and raise concerns about reverse causality—where elevated metal levels may result from pre-existing kidney damage rather than directly causing it. In addition, the exclusion of studies from some meta-analyses, coupled with selection biases and varying analytical approaches, further complicates the consolidation of findings.

Biomarkers used for exposure and kidney effects pose additional challenges. For Cd, urinary cadmium is the primary biomarker for long-term exposure, yet its reliability can be affected by kidney disease, urine dilution, and recent changes in Cd exposure, such as smoking or occupational sources. Similarly, Hg nephrotoxicity is often assessed with urinary mercury, but variability in its quantification and external contamination risks undermine its utility. For Pb and Cd, effect biomarkers such as β 2-M and NAG are considered early markers of kidney damage, yet their sensitivity under low-level exposures or normal physiological variability can lead to inconsistent interpretations. GFR estimates, when based on creatinine and widely used for Pb and Cd effects, may underestimate renal dysfunction due to the lack of adjustments for muscle mass or alterations in kidney physiology. Since confounding factors as diet can affect TAs levels, we decided to use to base our HBM-TV on TRA. However, measurement of TAs, iAs, or its metabolites varies by study, leading to challenges in standardising exposure estimates.

Individual susceptibility to metal toxicity varies greatly and is influenced by demographics (age, gender), lifestyle (smoking), genetic factors, and pre-existing conditions, such as diabetes. For example, diabetics may be more susceptible to Cd toxicity, and with the global rise in diabetes prevalence, the dose-response relationship for Cd and Pb may shift, warranting further investigation. Genetic polymorphisms also play a key role; for Hg and As, variations in genes related to metabolism or oxidative stress repair mechanisms affect individual responses, introducing another layer of uncertainty. Additionally, lifestyle factors such as smoking significantly amplify systemic Cd and As burdens, confounding associations between exposure and renal effects.

6.4.3. Uncertainties in exposure

Historical and regional variations in exposure further complicate risk assessment. In older occupational studies on Cd and Pb, observed effects may reflect past high exposures that no longer represent current environmental levels, risking overestimation of health risks. Conversely, the "healthy worker effect" in occupational settings may underestimate risks by selectively including healthier individuals. For As, exposure variability across regions (e.g., low level contaminated drinking (mineral) water or dietary intake from fish or rice) exacerbates the challenge of generalising findings to broader populations, particularly given the complex metabolism of arsenic species and varying health risks based on exposure profiles.

Moreover, the use of HBM data has the advantage of providing an estimate of aggregate internal exposure. However, for metals with a short half-life, such as As or Hg, the measuring of a single time point may introduce uncertainties in the exposure estimate. In addition, differences between the biomarkers measured and the hazard data require the use of conversion factors (for TAs or Pb), which introduce uncertainties.

Finally, co-exposure to other metals with neurotoxic effect (e.g., Cu, Li, Ni) remains underexplored but is critical for understanding cumulative risks. Interactions between metals may amplify renal or systemic effects, yet most studies assess single metal exposures in isolation.

6.5. Future perspectives

The MRA results obtained with HBM data can be compared with external MRA to validate the proof-of-concept. In Sprong et al. (2023) the authors performed chronic dietary mixture risk assessments for the same four metals, regarding the same effect and using the same approach. At the first glance the results (mRPI order of magnitude and risk drivers) seem consistent.

The proposed MRA strategy was a lower tier approach. The lower tier approach in risk assessment lacks refinement and specification of uncertainties. For example, there are ranges used as TV, not all metals are measured in the same matrix, and biomarker of effects differ for the metals. Therefore, whenever the lower tier mRPI is higher than 1, a second (or higher) tier is deemed necessary.

For the higher tier of risk assessment, the relative potencies of the hazards of the mixture components can be determined (hazard potencies relative to an index compound, Section 7.1). This will increase the accuracy of the risk assessment, because the contribution of each metal is weighted based on their relative potency. These RPFs should be based on a specified common adverse effect determined for the various mixture components in one study (in a common biological matrix) or in different studies with the same study design. For example, in case of nephrotoxicity, the glomerular filtration rate (GFR or eGFR) could be determined in a study (or similar studies) in

a specified (sub)population in which also the various metals (including their metabolites) have been determined. The summed internal exposure equivalents are compared to the HBM-TV of the index compound. For the selected four metals (As, Cd, Hg and Pb) these RPFs are not available yet.

7. Mixture risk assessment: PFAS and immunotoxicity

7.1. Mixture risk assessment strategy

There is a growing concern regarding the effect on human health of the PFAS chemical class. PFAS are highly persistent, mobile, bioaccumulative, and ubiquitous in the environment. Immunotoxicity is considered as one of the critical health effects (EFSA CONTAM Panel et al., 2020). HBM data, reflecting real-life exposure to chemicals, commonly shows that individuals are exposed to more than one PFAS simultaneously. PFAS were measured in blood (serum or plasma), which is a suitable exposure metric for substances with a long biological half-life such as certain PFAS of interest for risk assessment (Calafat et al., 2019). The strategy applied in this case study to perform MRA is based on RPFs. These factors express the toxicological potency of a substance compared to the potency of a reference substance for a common effect. In this case the RPFs derived by Bil et al. (2023) are used to express the toxicological potency of each PFAS compared to the potency of PFOA based on their immune effects. The individual internal exposure level (i.e., HBM blood concentration) of a PFAS is multiplied by the corresponding RPF value (Equation j). The obtained values are the PFOA equivalent (PEQ) blood concentrations, that after they are summed (Equation k) represent mixture exposure in PEQs. A Risk Characterisation Ratio (RCR) is established by comparing sum PEQs in the population to an HBM-GV (Equation l). In case the RCR is below 1, a potential risk to adverse health effects can be considered negligible. If the RCR is equal to or above 1, it means that a potential health risk cannot be excluded (PARC, 2023).

Equation j

$$PEQ = EL_s \times RPF_s$$

Equation k

$$\text{sum PEQ} = \sum_{s=1}^S EL_s \times RPF_s$$

Equation l

$$RCR = \frac{\text{sum PEQ}}{HBM-GV}$$

MRA was performed on four different subpopulations divided by age and/or gender: women-of-childbearing-age (18-45 years), children (3-17 years), adults (>18 years), and workers. Two different treatments for censored data were applied (see Section 7.2.2) ending in 8 scenarios in total (Table 6).

Table 6. Overview and numbering of applied scenarios to perform MRA for PFAS and immune effect.

Scenario	Population				Treatment of censored data	
	Women of childbearing age (18-45 y)	Children (3-17y)	Adults (>18y)	Workers	LB (By 0 or by f* LOQ)	UB (By f* LOD or by LOD + f* (LOQ - LOD))
1	X					X
2		X				X
3			X			X
4				X		X
5	X				X	
6		X			X	
7			X		X	
8				X	X	

7.2. Data

7.2.1. Hazard data

The internal RPFs used in this analysis have been derived for hepatotoxicity and immunotoxicity effects showing strong correlation between both effects (Bil et al., 2022, 2023). Pooled geometric means and pooled variances, and corresponding P5, P95 and geometric standard deviations were obtained using RPFs based on absolute thymus weight, relative thymus weight, absolute spleen weight, and relative liver weight (Bil et al., 2023) and on relative liver weight (Bil et al., 2022). This hazard data allows for inclusion of nine PFAS in our MRA. The pooled RPF distributions for nine PFAS are summarised in Table 7.

Table 7. Pooled (lognormal) distribution of the internal RPF distributions for PFAS based on several immunotoxic and hepatotoxic effects.

Substance	Geometric mean	P5	P95	Geometric Standard Deviation
PFBA	1.34	0.924	1.95	1.25
PFHxA	7.35	5.24	10.3	1.23
PFOA	1	-	-	-
PFNA	5.84	4.17	8.18	1.23
PFDA	8.15	5.31	12.5	1.30
PFDoDA	11	8.64	14	1.16
PFBS	0.986	0.661	1.47	1.28
PFHxS	0.503	0.347	0.73	1.25
PFOS	3.79	2.66	5.38	1.24

The HBM-GV used in the MRA is based on EFSA work to derive a group tolerable weekly intake (TWI) of 4.4 ng/kg bw/week for a sum of four PFAS (PFOA, PFNA, PFHxS and PFOS). One of EFSA steps to derive the TWI by extrapolating the BMDL10 was a modelled serum level of 6.9 ng PFAS/mL in mothers at the age of 35 (EFSA CONTAM Panel et al., 2020). This value should protect the mother and their children taking into account breastfeeding. EFSA assumed equipotency among the four studied PFAS. The PBK model applied to get the internal blood values modelled the concentration overtime (from birth to 35 years) to reach the value of 6.9 µg/L by the age of 35 (Figure 9). The overtime modelling of the serum level is used as an age-dependant HBM-GV for PFOA in the MRA.

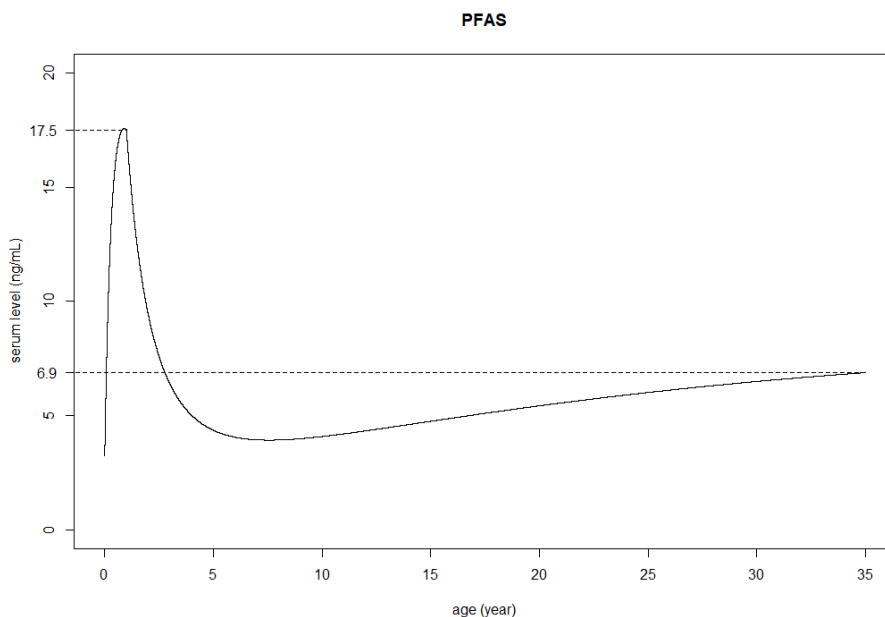


Figure 9. Reproduced sum PFAS blood levels in women of reproductive age over time, using the PBK model codes for PFOA and PFOS in Appendix M of the EFSA opinion (2020).

7.2.2. Human biomonitoring data

Studies analysing at least two of the targeted PFAS in blood for general population or workers have been selected for this case study. Overall, 22 studies from 11 countries and 11 institutes have been used (Figure 10 and Table 8). The sampling period goes from 1996 to 2021. The size of the studies was between 30 and 1533 individuals. PFAS were measured in blood plasma or serum. From 2 to 9 PFAS were analysed by datasets. For the children sub-group 10 datasets were used to perform the analysis. For adults and women-of-childbearing-age, 10 and 11 datasets were included, respectively. Moreover, one dataset for workers was used.

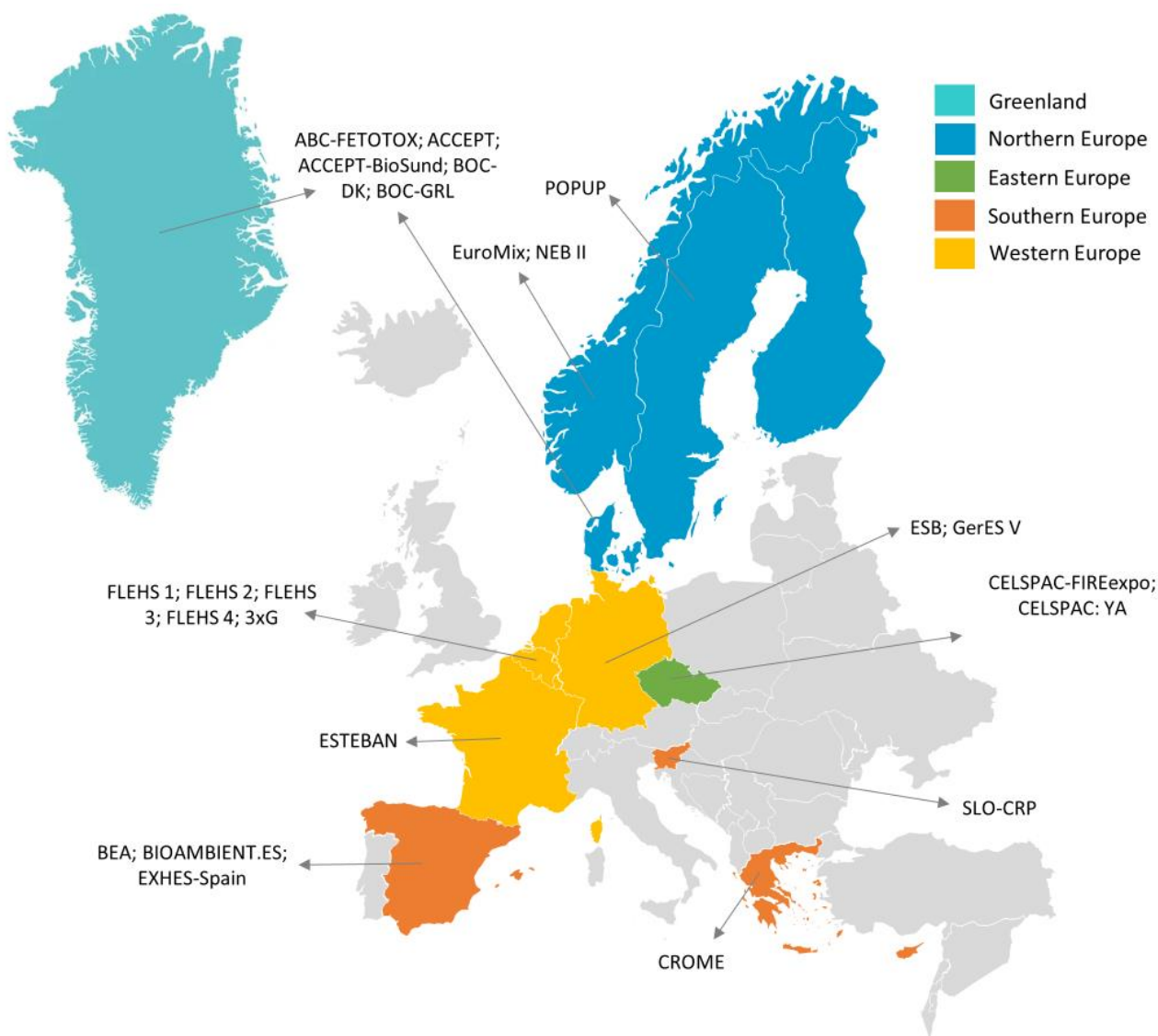


Figure 10. An overview of the HBM datasets applicable for the PFAS - immunotoxicity case study which were organised and analysed in the MCRA toolbox within the PARC project Real-life mixtures. Per country, the study name(s) are provided.

Regarding the treatment of left censored data, two treatments were applied to left censored data (i.e., below the LOD and LOQ values). For the lower bound (LB) scenario, censored values below the LOD or LOQ were respectively substituted by the 0 and LOD value. For the upper bound (UB) scenario, values below the LOD or LOQ were respectively substituted by the LOD and LOQ value. A summary of the scenarios can be found in Table 6.

Table 8. PFAS & immunotoxicity case study – HBM datasets.

Survey	Country/region	Institute	Sampling year	Biological matrix	Survey type*	Population	Age (P25-P75)	N	No. PFAS
3xG children	Belgium	VITO	2019-2021	Serum	GP	children	3.5 - 3.5	173	9
ACCEPT	Greenland	AU-PH	2010-2015	Serum	PW	women	24 - 31	495	8
ACCEPT-BioSund	Greenland	AU-PH	2019-2020	Serum	GP	women	30 - 37	94	8
						adults	30.5 - 39	166	8
BEA	Spain	ISCIH	2017-2018	Serum	GP	children	14 - 15	272	8
BIOAMBIENT	Spain	ISCIH	2009-2010	Serum	GP	women	29 - 39	294	5
						adults	32 - 47	749	5

BOC-DK	Denmark	AU-PH	1996-2002	Serum	PW	women	27 - 33	412	8
BOC-GRL	Greenland	AU-PH	2000-2003	Serum	CC	adults	44.5 - 60	146	6
			2011-2014	Serum	CC	adults	44 - 60.2	99	8
CELSPAC-FIREexpo	Czech Republic	RECETOX, MU	2019-2020	Serum	W	workers	23 - 30	110	8
CELSPAC-YA	Czech Republic	RECETOX, MU	2019	Serum	GP	women	27 - 27	149	8
						adults	27 - 28	291	8
CROME	Greece	AUTH	2020-2021	Serum	GP	children	12 - 15	52	8
ESB	Germany	UBA	2015-2019	Plasma	GP	women	21.5 - 24	30	9
						adults	22 - 24	60	9
ESTEBAN	France	Anses	2014-2016	Serum	GP	women	32 - 42	131	9
						children	9 - 15	246	9
						adults	42 - 62	746	9
EuroMix	Norway	NIPH	2016-2017	Serum	GP	adults	33 - 50.2	143	8
EXHES-SP	Spain	IISPV	2016-2017	Plasma	PW	women	30 - 39	74	2
FETOTOX	Denmark	AU-PH	2008-2013	Serum	PW	women	27 - 31	1533	8
FLEHS 2	Belgium	VITO	2008-2009	Serum	GP	women	31 - 38	107	2
						adults	30 - 37	201	2
			2010-2011	Serum	GP	Children (Menen)	14 - 15	197	2
FLEHS 3	Belgium	VITO	2014	Serum	GP	adults	54 - 61	205	5
FLEHS 4	Belgium	VITO	2017-2018	Serum	GP	children	14 - 15	410	8
GerES V	Germany	UBA	2014-2017	Plasma	GP	children	8 - 14	1,083	9
NEB II	Norway	NIPH	2016-2017	Plasma	GP	children	12 - 13	177	7
POPUP	Sweden	SLU	2015-2019	Serum	GP	women	29 - 37	203	8
						children	8 - 12	59	8
SLO-CRP	Slovenia	JSI, NIJZ	2018	Serum	GP	children	13 - 14	94	8

* GP: General population, PW: Pregnant women, CS: Case-control, W: Workers.

7.3. Mixture risk assessment results

The MRA strategy developed for PFAS and immunotoxicity was applied to 22 HBM datasets across Europe. In all datasets analysed, there were a proportion of individuals exceeding the RCR threshold of 1 to a greater or lesser extent. The percentage of individuals with a RCR exceeding the threshold value of 1 ranged from 72% to 100%. For children, the range of the median RCR of the analysed datasets ranged from 1.6 to 6.29, for adults (including workers) from 2 to 25, and for women-of-childbearing-age from 1.45 to 18.40. It is important to note that the datasets are very heterogenous (e.g., different PFAS measured, different sample sizes, different sampling periods, etc.), therefore, making it difficult to compare results between countries.

The risk drivers of the PFAS mixtures in the populations children, women-of-childbearing-age and adults are presented in Figure 11, Figure 12 and Figure 13, respectively. Whereby the percentage contribution of the individual PFAS to the overall RCR for the upper bound scenario is shown. The lower bound scenario results are not presented. For the women-of-childbearing-age group, one case-control dataset and three pregnant women datasets were included in the analysis alongside the general population datasets. For the adult group, two case-control datasets and one occupational dataset were included in the analysis alongside the general population datasets. PFOS was found to be the risk driver of the PFAS mixture regardless of the studied population due to its RPF combined with general higher observed exposure levels in the HBM datasets. It was followed by PFNA, PFDA, PFOA and PFHx depending on the dataset.

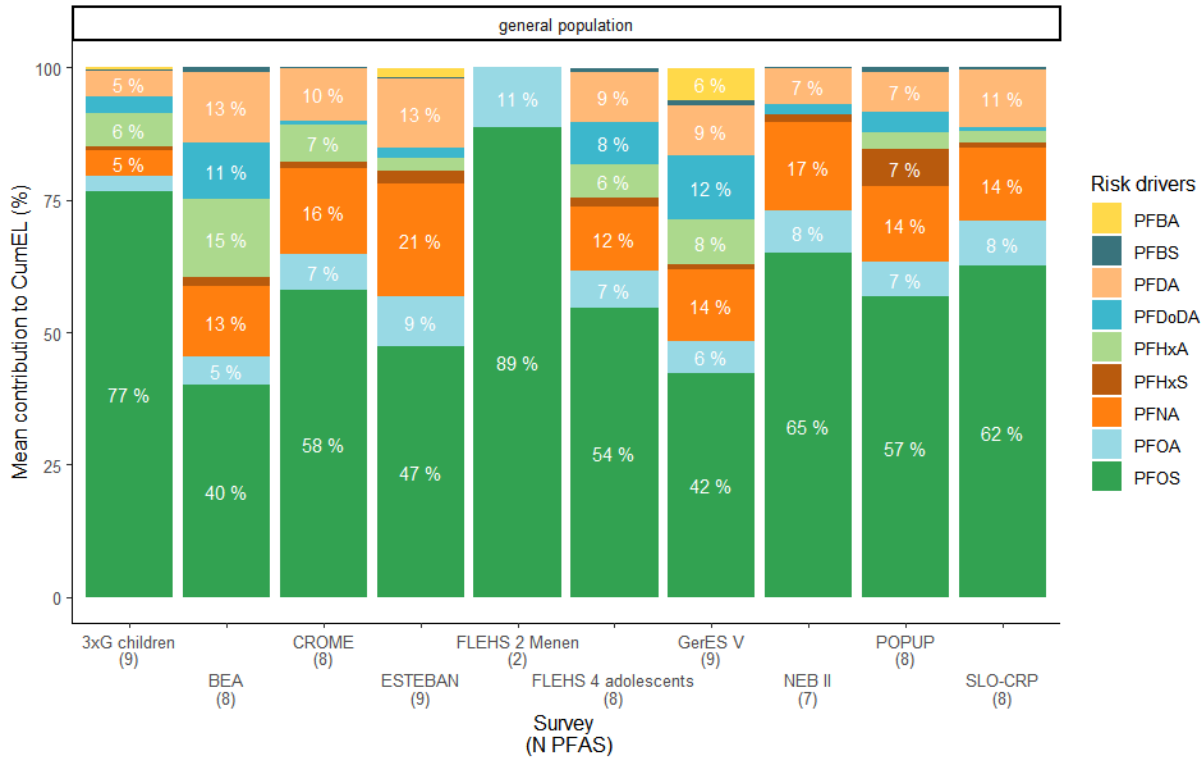


Figure 11. Individual PFAS contributions to the overall RCR in children (upper bound scenario).

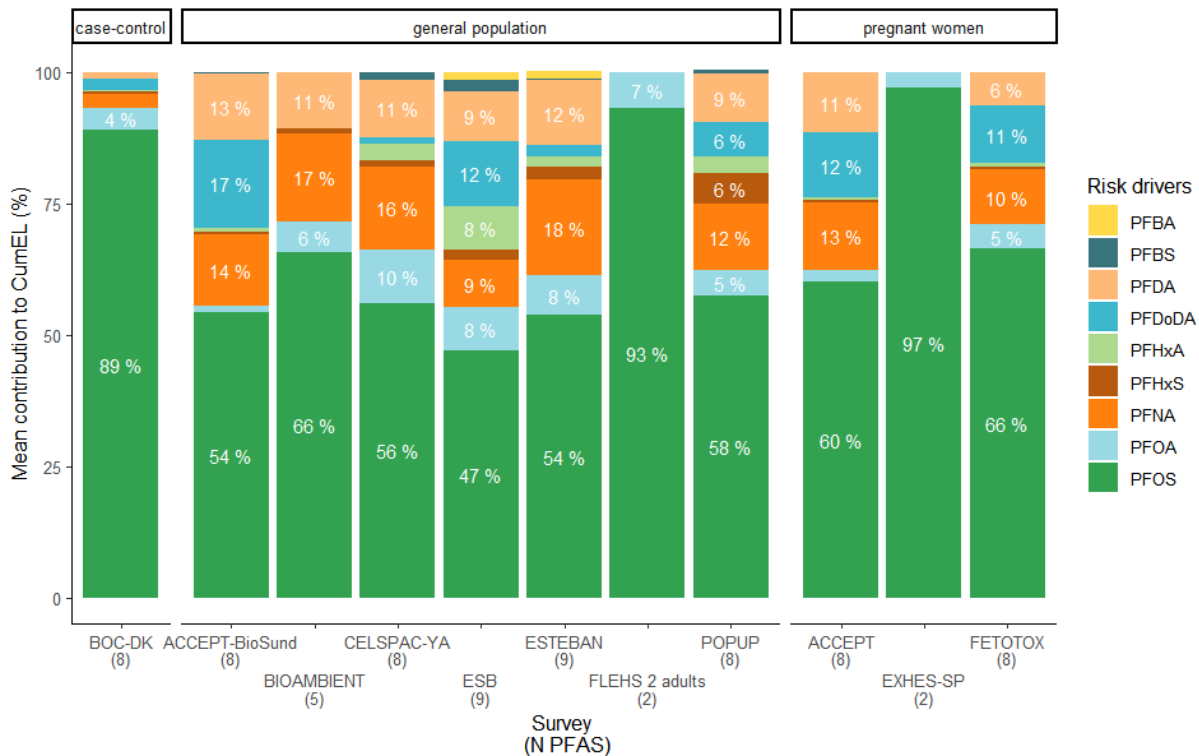


Figure 12. Individual PFAS contributions to the overall RCR in women-of-child-bearing-age (upper bound scenario).

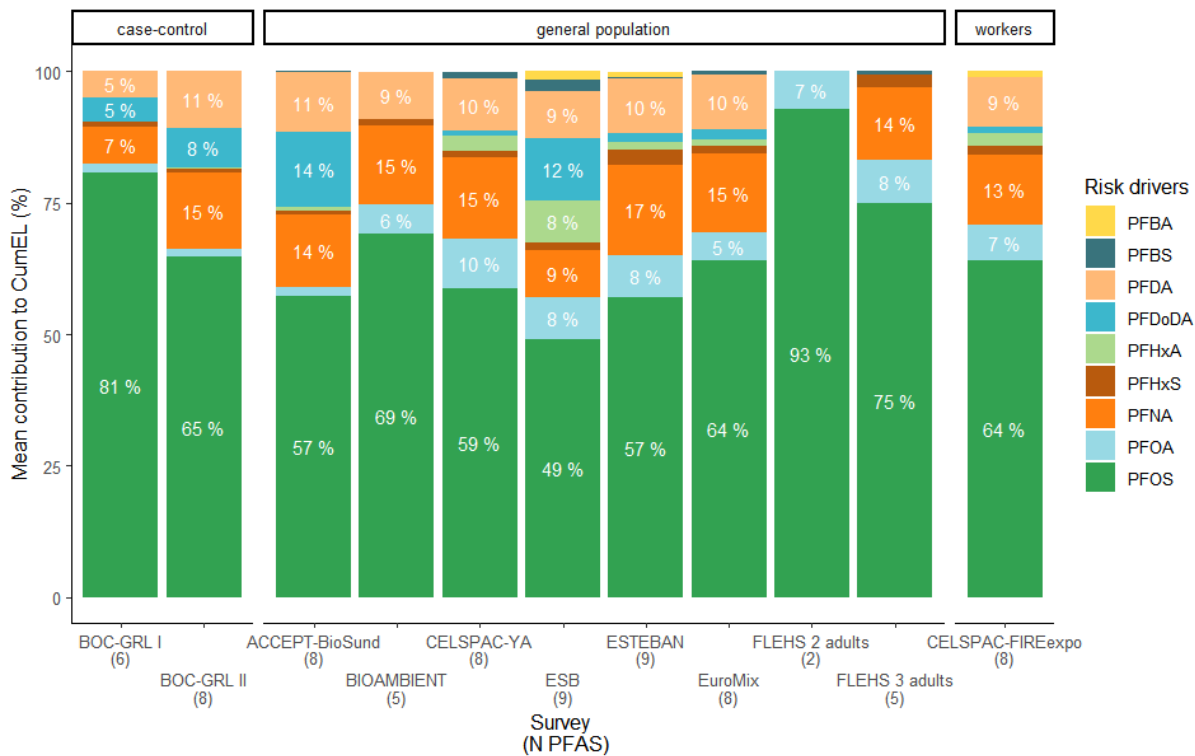


Figure 13. Individual PFAS contributions to the overall RCR in adults (upper bound scenario).

7.4. Uncertainties

7.4.1. Uncertainties in the hazard assessment

EFSA (2020) noted that the stronger association for PFOA compared to PFOS as indicated by the human epidemiological data, conflicted with the higher potency that is seen for PFOS compared to PFOA in various animal studies. Studying relative potencies in epidemiological studies is difficult in practice, as the serum concentration of one PFAS may be highly correlated with the serum concentration of other PFAS. As long as determination of relative potencies for PFAS based epidemiological studies remain a challenge, experimental animal studies can be used to verify if an assumption on equipotency is likely, even if this approach suffers from the general issue of species differences.

Ideally, RPFs for immunotoxicity should be derived based on functional immunotoxicity assays performed using the same test protocol, e.g., a T-Cell Dependent Antibody Response (TDAR) assay under similar testing conditions (Bil et al., 2023; Vandebriel et al., 2024). The antigen-specific IgM and/or IgG serum concentrations measured in the TDAR assay after antigenic challenge should be treated as a considerably more precise measure of the adaptive immune response compared to total serum globulin concentration or lymphoid organ weight changes in unchallenged animals. However, since such information is not available for PFAS at the moment, we derived RPFs based on spleen and thymus weight decrease. The results of our study fit to the overall picture that PFAS cause immunosuppression and degeneration of lymphoid tissues. There is ample evidence from animal toxicity experiments illustrating that exposure to PFAS causes atrophy of the spleen, thymus, and lymph nodes, results in bone marrow cell depletion, decreases the TDAR and the T-Cell-Independent Antibody Response (TIDAR), reduces the host resistance to infections, leads to decreased natural killer cell activity, and reduces clonal production of leukocytes (Ehrlich et al., 2023).

The set of internal RPFs is based on PFOA as index compound. Ideally, this approach should include a “RPF adjusted” serum concentration expressed in PFOA equivalents (PEQs) as point of departure, as basis for the HBM-GV. However, due to practical reasons (i.e., unavailability of the raw, individual data in the public domain), we cannot adjust the POD to PEQs ourselves. Consequently, in the current assessment, we use the age dependent HBM-GV and compare the PEQ exposure to this sum-4 value, allowing the sum value for PFAS to be filled up by PFOA

(equivalents) only (RIVM, 2021). We make this assumption for two reasons 1) EFSA assumed equipotency among the substances in the mixture and 2) Abraham et al. (2020), the study EFSA based its risk assessment on, concluded that there is only an association with PFOA, and not with the other three PFAS. PEQ distributions of the populations of interest are compared to the age-dependent HBM-GV to interpret the risk on detrimental health effects in these populations. This introduces uncertainty around the risk estimate. We intend to use the HBM-II value for PFOA by the German HBM Commission (Schümann et al., 2021) as additional toxicological value to the age-dependent HBM-GV based on the EFSA group TWI. We do this in order to illustrate what implications the choice of the HBM-GV, and the assumptions needed to make based on this value in combination with internal RPF distributions, has on the MRA outcomes.

7.4.2. Uncertainties in the exposure assessment

In our case study, any PFAS with HBM data available is considered in scope for the purpose of MRA. There are over 37 PFAS biomarkers (not even differentiating between linear and branched PFAS forms) in the biomarker list (Section 4.1). Nevertheless, in order to be able to perform quantitative MRA, we need sufficient hazard data for these chemicals as well. As we base our risk assessment primarily on *in vivo* data available, it resulted in inclusion of nine PFAS in our MRA case study. This does not mean that exposure to the other PFAS is negligible. Because we could not incorporate these PFAS in our risk assessment, it inevitably means we underestimate the cumulative risk to a certain, unknown, extent.

Whereas in some datasets both short- and long-chain PFAS were analysed in blood samples, detection rates for substances like PFBS, PFPeA, and PFHxA were generally low. This could partly be due to high LOD/LOQ in certain studies, but it may also reflect the limitations of using blood as the exposure matrix for some PFAS. Short-chain PFAS have shorter half-lives in humans, ranging from days to months, unlike long-chain PFAS, which persist for years (EFSA CONTAM Panel et al., 2020). Given their faster elimination, urine could be a supplementary matrix for evaluating exposure alongside blood samples. Expanding on this research under the PARC initiative would provide valuable insights into how these substances are best monitored in humans.

7.5. Future perspectives

The case study presented here is a proof-of-concept for the use of HBM data in retrospective MRA and is not intended to illustrate risks from the substances analysed. In our case study, we anticipate that between 72-100% of the individuals in the studied populations exceed the HBM-GV, with RCRs varying between 1.6-25 depending on the studied population. When populations exceed such threshold value, it means that there is a concern regarding exposure to PFAS mixtures, specifically for the most sensitive sub-populations that the toxicological value aims to protect. Exceedance of this threshold does not give specific insights in what type of health effects may start appearing in the population at the observed exposure levels, in which populations, and what the degree in severity of these health effects is. To inform on such aspects, a health impact study should be performed. In P6.2.4, a health impact case study started on PFAS and immune health, that will shed some more light on this in the near future.

The highest blood serum or plasma concentration data in all datasets and across all age groups is PFOS, and PFOS is also the largest contributor to the risk ratio distribution. Blood concentrations, that reflect aggregate exposure from different sources, cannot inform on source contribution in isolation. However, it is insightful to have a better understanding of the sources that contribute to the aggregate exposure to PFOS and other main contributors, to inform and steer effective policy making to reduce exposure to PFAS. This can be done by combining source-to-dose modelling and aggregate exposure modelling, two activities performed under P6.2.1.

In WP4, there are large, recent biomonitoring studies underway to deliver new information on PFAS exposure in the European population, across generations and geographical regions. With the knowledge of the HBM4EU project in hand, the follow-up HBM studies in PARC will have a standardised and protocolled character. WP4 includes PFAS under Aligned Studies (P4.1.1.2) for teenagers (12–17 year old) and adults. Additionally, a dedicated project under P4.1.1.3 involves PFAS for children (6–11 year old), and under P4.1.1.4, PFAS are addressed in workers. This means that the current uncertainty in this risk assessment case study, being that of the heterogeneity in datasets used (with regard to study populations, timepoint of sampling, analytical methods, etc), will be largely reduced when we will be able to use the new data from PARC WP4.

In PARC WP5, several NAM (New approach methodology)-based methods are being developed to improve our understanding of the impact of chemical exposure on the immune system (Snapkow et al., 2024). One of the prioritised chemical groups to work on in WP5 is PFAS. We hope to be able to integrate insights of this WP in our MRA approach in the future. Nevertheless, using *in vitro* data for derivation of RPFs for PFAS is complicated. The combination of the intrinsic toxicity and the bioaccumulation potential together determine the potency of a PFAS. Consequently, RPFs based on external intake doses are not the same as internal (systemic) RPFs based on blood concentrations, or *in vitro* RPFs. When one foresees to use *in vitro* information as the basis for derivation of internal RPFs to be applied in MRA using blood data, a quantitative *in vitro-in vivo* extrapolation (QIVIVE) should be incorporated as well to account for differences in accumulation potential among PFAS over time. The PBK models needed for this are now developed and standardised for a set of PFAS in P6.2.2. However, such work would be very valuable, we would, in parallel, encourage performing standardised TDARs on a broad range of PFAS within PARC WP5. As described above, such studies are very informative for deriving RPFs directly related to immunotoxicity. Besides that, it also potentially increases the number of PFAS that can be taken up in scope of the MRA.

There are many substances with exposure information for which there is currently insufficient information on the (immunotoxicity) hazard, one of the reasons being that functional immunotoxicity testing is hardly covered within our EU regulatory frameworks at the moment (Vandebriel et al., 2024). In the next phase of the project, we aim to include substances with immunotoxicity concern outside the PFAS group as well, to extend our immunotoxicity case study with real-life mixtures that exceed confined chemical groups.

8. Mixture risk assessment: metals and developmental neurotoxicity

8.1. Mixture risk assessment strategy

Developmental neurotoxicity (DNT) has been recognised as a major public health concern by policy-makers worldwide (Grandjean & Landrigan, 2014), and references therein), with reduction in IQ (Intelligence Quotient) being the most well-documented and widely studied outcome. Mechanistically, DNT is the result of disruptions to critical processes of neuronal development during vulnerable periods of early life. Although neurotoxic effects can occur throughout life, impairment of IQ has been primarily linked to chemical exposures occurring from in utero development through adolescence and early adulthood (early 20's). These exposures can impact various neurodevelopmental processes such as neurogenesis, synaptogenesis, and myelination (Black et al., 1998; Greenough et al., 1987). The most sensitive populations are therefore considered to be pregnant women (i.e., the developing foetus) and children.

In this proof-of-concept exercise, three frequently monitored metals which also have been determined to cause potential IQ-loss as an adverse effect were chosen: lead (Pb), methylmercury (MeHg) and toxicologically relevant arsenic (TRA) (Smirnova et al., 2014). Human exposures to these three heavy metals are known to be due to both naturally-occurring contamination and anthropomorphic activities, and understanding the human exposures and the potential risks from those exposures can help prioritise and inform policy initiatives around these three regulated contaminants.

Pregnant women and children are considered to be the most sensitive populations for effects on neurodevelopment. Therefore, the datasets have been divided by age groups for the analysis: children (3-9 years), adolescents (10-17 years), and women-of-childbearing-age (18-45 years).

Similar to the metals and nephrotoxicity case study (see Section 6.1), the mRPI approach was used to perform the MRA. The ratios of the exposure level from HBM data and HBM-TVs are added together to calculate the mRPI (Equation m). The obtained mRPI value is compared to 1 which indicates the cumulative exposure might be concerning regarding the targeted effect.

Equation m

$$mRPI = \frac{EL_{TRA}}{HBM TV_{TRA}} + \frac{EL_{MeHg}}{HBM TVP_{MeHg}} + \frac{EL_{Pb}}{HBM TV_{Pb}}$$

Where:

mRPI= modified Reference Point Index;

EL=exposure level from HBM data;

HBM-TV=Human Biomonitoring-Toxicological value.

The mRPI method is able to estimate the combined risk from substances measured in different matrices. Assuming the ratio is correct for each substance, it should adequately represent the potential risk from the internal exposures measured for individuals in the HBM studies for the metals in question, despite the fact that the measurements were performed in different body matrices. The mRPI value is compared to 1 informing that represent the case where the exposure is higher than the hazard threshold. Thus, if a mRPI is above 1 it means that there may be concern regarding exposure risk cannot be excluded.

8.2. Data

8.2.1. Hazard data

Internal reference values for the various metals were derived from epidemiological data. The biological matrix from which these values were derived was an important determinant of the inclusion of specific HBM studies in this project. HBM studies that measured the metal in a different matrix were included only if conversion between matrices for the metal in question was possible. In this case, effects on IQ development are the most potent effect of both lead and methylmercury, and thus HBM-GVs are used. For toxicologically relevant arsenic (TRA), effects on IQ development are not the most sensitive, and thus an HBM-TV was derived using the BMDL5 of data from Vahter et al., (2020) (the key study for DNT according to EFSA, (2024)). The HBM-TVs and HBM-GVs for developmental neurotoxicity (DNT) used in this case study are shown in Table 9. UFs are not applied, as the data were derived based on numerous human data in the critical populations, except in the case of the conversion from hair to blood for methylmercury, where a UF was added to cover the uncertainties in this calculation (2 for variation in the hair to blood ratio and 3.2 for individual toxicokinetic variations). These are all aligned with the assessments of EFSA for the various metals.

Table 9. Metals & DNT case study – Hazard data.

Metal	Type of data	Matrix	HBM-GV/ TV (UF)	Population	Reference
Pb	Human - Epi	Blood	12 µg/L (UF =1)	Children	EFSA 2010
MeHg	Human - Epi	Hair → Blood	5 µg/L (UF = 6.4)	Pregnant women	US-EPA 2001
MeHg	Human - Epi	Hair	8 mg/kg (UF = 1)	Pregnant women	US-EPA 2001
TRA	Human - Epi	Urine	16.1 µg/L (UF = 1)	Children	Vahter, et al. (2020)/ (EFSA, 2024)

8.2.2. Human biomonitoring data

The selected datasets for this case study were required to have concentration data of at least two metals in the targeted biological matrix measured in children (3-9 years), adolescents (10-17 years) or women-of-childbearing-age (18-45 years). Figure 14 shows the selected 19 datasets for this case study. The subgroup women-of-childbearing-age (hereinafter referred to as “women”) includes the most datasets (12), followed by seven for adolescents and two for children. Most studies include data for only one subgroup, with the exception of ESTEBAN and GERES V, which were divided into three and two datasets, respectively, for subgroup analysis. Table summarises the different biomarker of exposure-biological matrix pairs selected in this case study. Regarding the biomarkers of exposure, similarly as the metals and nephrotoxicity case study for As, for As, TRA and TAs in urine were selected. TRA (the sum of As(III), As(V), MMA and DMA) is the selected biomarker, thus similarly as the metals and nephrotoxicity case study TAs is converted in TRA by a conversion factor. For Pb, concentrations of Pb in hair or whole blood or urine were selected. For MeHg, concentrations of MeHg in hair or whole blood were selected.

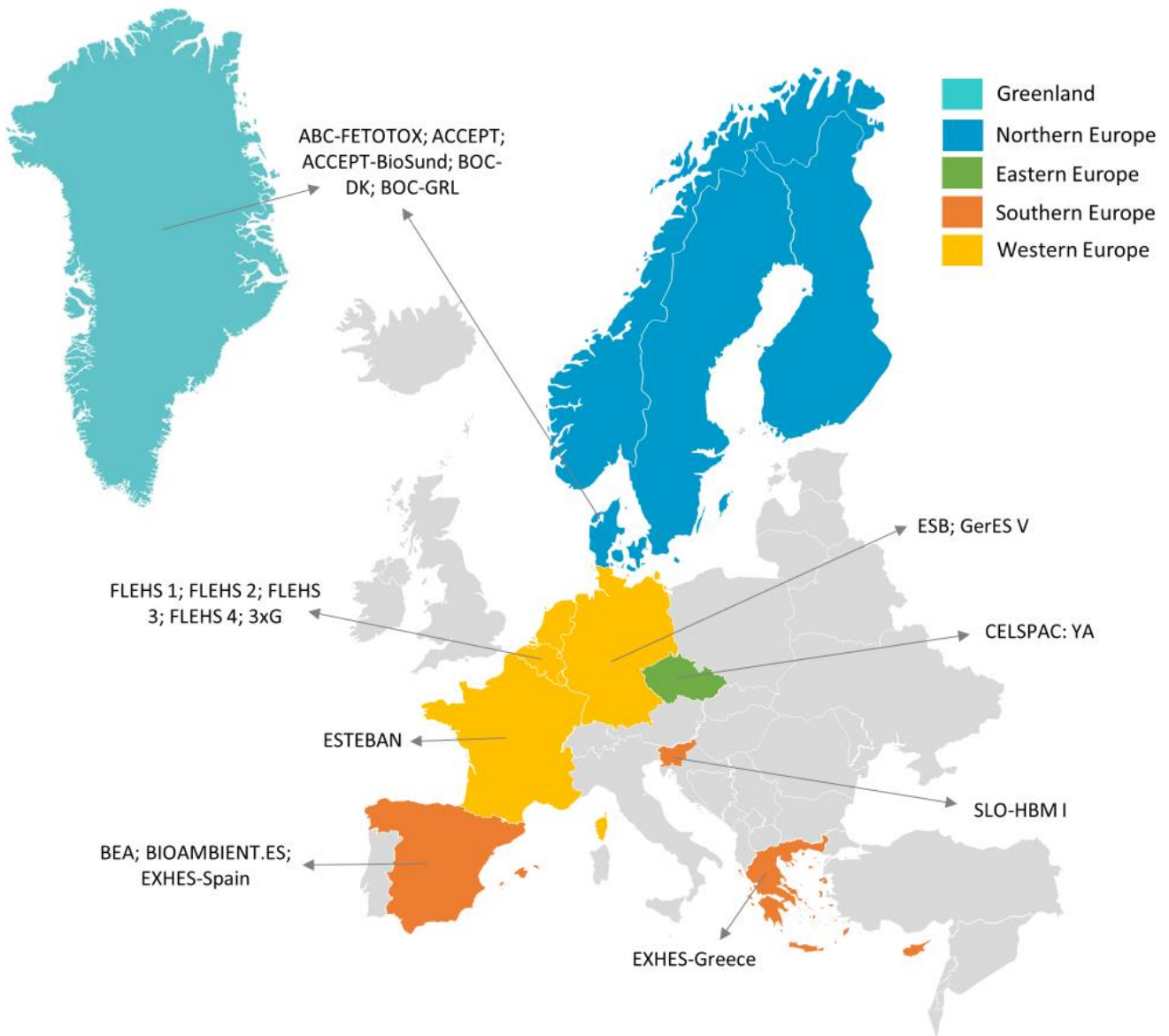


Figure 14. An overview of the HBM datasets applicable for the Metals - DNT case study which were organised and analysed in the MCRA toolbox within the PARC project Real-life mixtures. Per country, the study name(s) are provided.

Table 10. Heavy metals & DNT case study - biomarker of exposure-biological matrix pairs.

Heavy metal	Biomarker	Biological matrix
Arsenic	TRA or Total As	Urine
Lead	Pb	Blood or urine
Mercury	MeHg or Hg	Hair or blood or urine

As the HBM-GV in urine is based upon specific-gravity corrected values, for the best comparison the same correction was applied to the HBM measured values. Thus, the urinary biomarkers were adjusted using SG to account for urinary dilutions. The following formula was applied to calculate the biomarker data normalised (Equation n):

Equation n

$$C_{SG} = \frac{C \times (1.024 - 1)}{SG - 1}$$

With C_{SG} being the biomarker concentration in urine normalised for specific gravity, C the measured concentration of the biomarker in urine and SG the specific gravity, 1.024 was taken as average urinary concentration of specific gravity

Regarding the treatment of left censored data, two scenarios were applied to left censored data (i.e., below the LOD and LOQ values). For the lower bound (LB) scenario, censored values were substituted by 0. For the upper bound (UB) scenario, values below the LOD or LOQ were respectively substituted by the LOD or LOQ value. A summary of the scenarios can be found in Table 11.

Table 11. Overview and numbering of applied scenarios to perform MRA for metals and IQ loss.

Scenario	Population					Treatment of censored data	
	Infants < 1y	Toddlers (1-2y)	Older children (3-9y)	Adolescents (10-17y)	Women of childbearing age (18-45y)	LB (By 0 or by LOD)	UB (By LOD or by LOQ)
1	X						X
2		X					X
3			X				X
4				X			X
5					X		X
6	X					X	
7		X				X	
8			X			X	
9				X		X	
10					X	X	

8.3. Mixture risk assessment results

The MRA strategy developed for the metals and DNT case study was applied to 19 HBM datasets across Europe. Overall, in all datasets analysed, there were a proportion of individuals exceeding the threshold of 1 to a greater or lesser extent. For the datasets including the three target metals, the percentage of individuals with an mRPI exceeding the threshold value of 1 ranges from 73% to 96%. For children, the median mRPI is between 1.8 to 1.5, for adolescents between 0.46 to 1.8, and for women-of-childbearing-age between 0 to 9. It is important to note that the datasets are heterogenous (e.g., different metals measured (in varied biological matrixes), sample sizes, sampling periods, etc.), making it difficult to compare the results between countries (Section 8.4).

The risk drivers of the metals mixtures in the populations children, adolescents and women-of-childbearing-age are presented in Figure 15, Figure 16Figure 12 and Figure 17Figure 13, respectively, whereby the percentage contribution of the individual metals to the overall mRPI for the upper-bound scenario is shown. The lower bound scenario results are not presented. Regardless of the population type, where measured, Pb was most often found to be the risk driver of the mixture, followed by TRA as the second risk driver (where measured).

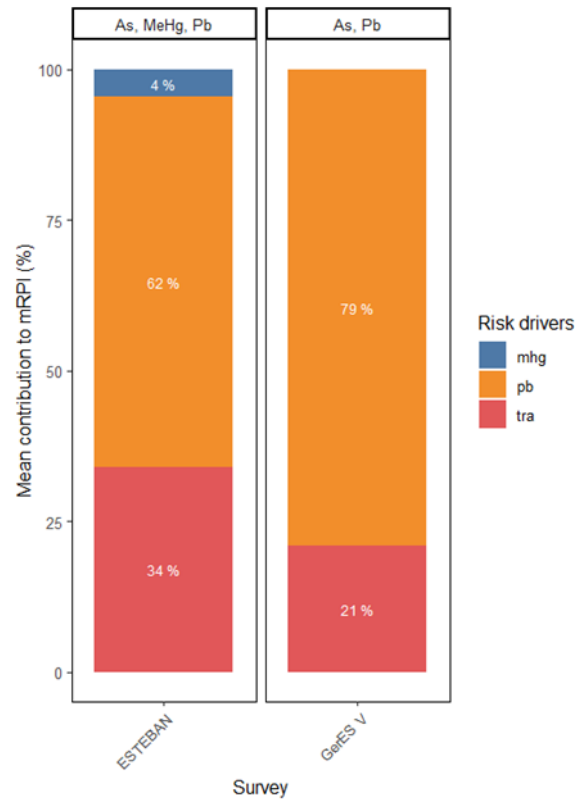


Figure 15. Individual metal contributions to the overall mRPI in children (upper bound scenario).

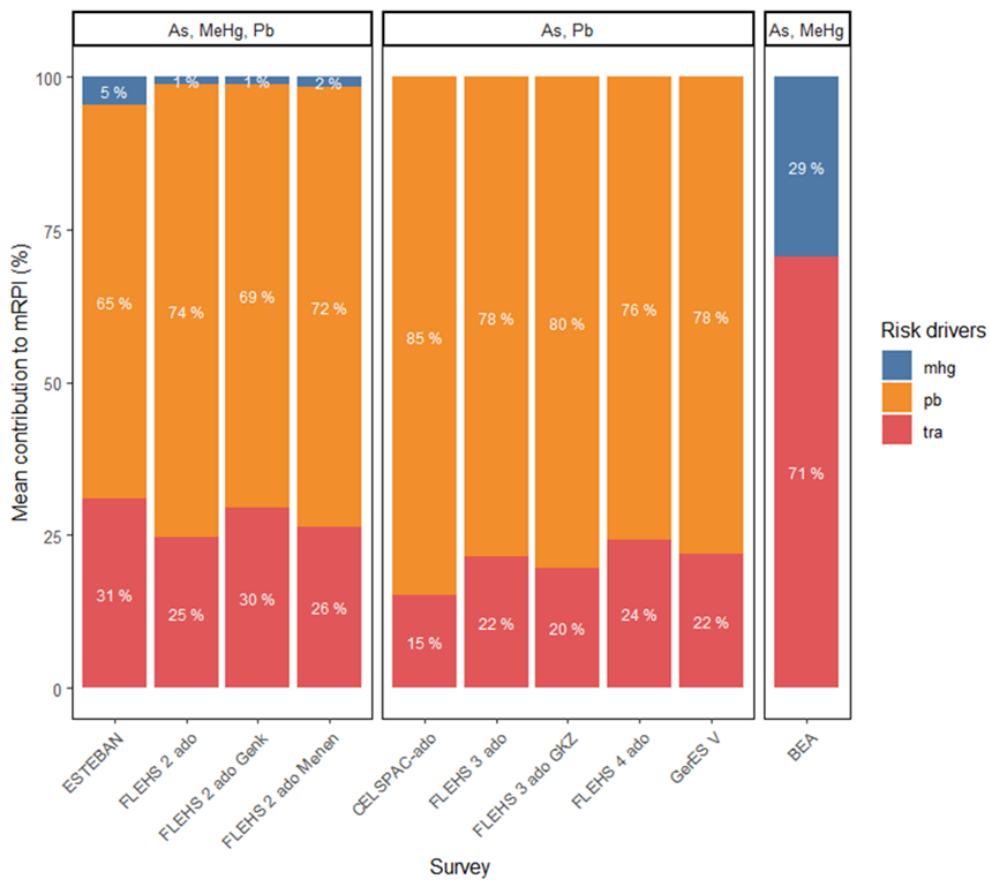


Figure 16. Individual metal contributions to the overall mRPI in adolescents (upper bound scenario).

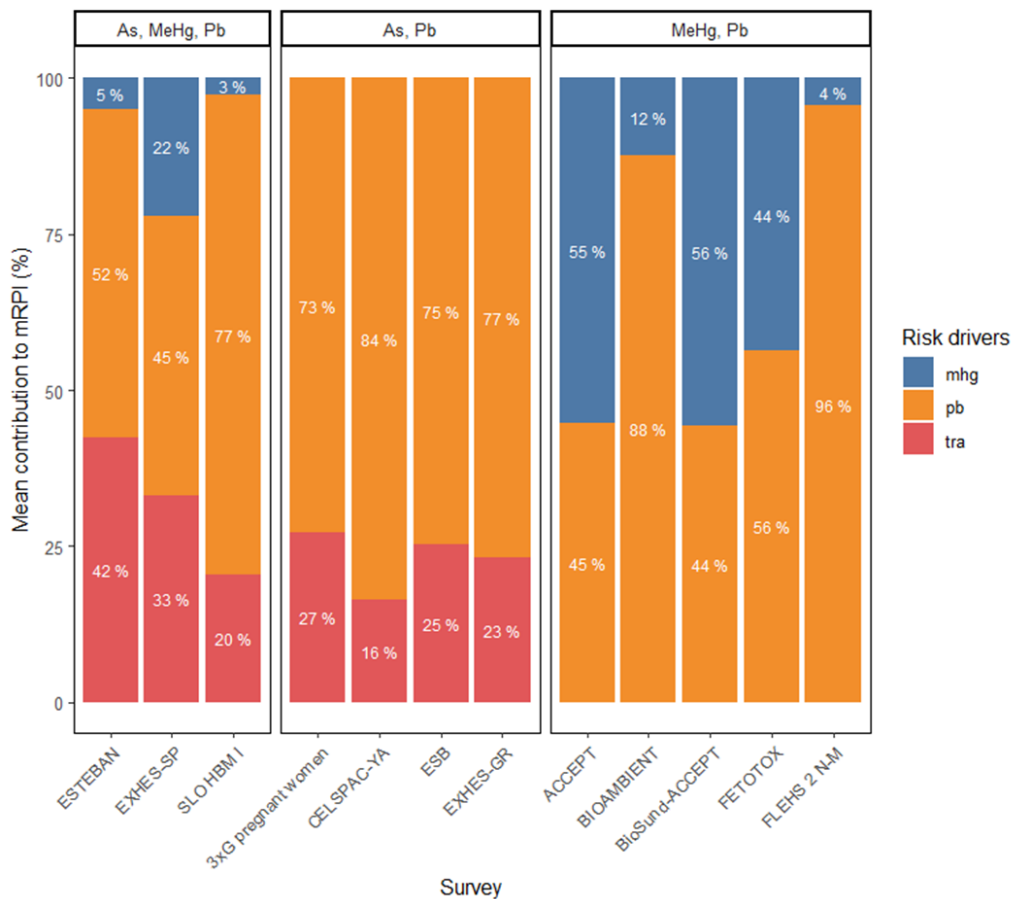


Figure 17. Individual metal contributions to the overall mRPI in women-of-child-bearing-age (upper bound scenario).

8.4. Uncertainties

8.4.1. Uncertainties in mixture risk assessment methodology

8.5 The approach developed is based on dose addition to estimate the potential for mixture toxicity. In most cases, dose addition is sufficiently conservative to appropriately estimate the potential for toxicity of the mixture (EFSA, More, et al., 2019). However, in some (notable) cases, synergism or potentiation have been noted (e.g., ergosterol biosynthesis inhibitors (EBI) fungicides, neonicotinoids) (Cedergreen, 2014; Hernández et al., 2017; Thompson et al., 2014). Types of synergism could include action at the same receptor or interaction in the same key event (toxicodynamic synergism) or when one substance changes the speed or outcome of the normal metabolic processing of another (toxicokinetic synergism). In this case, it is unlikely that toxicokinetic synergism/potentiation will happen, as synergism has only been observed at very high exposure levels (Cedergreen, 2014). However, adding toxicokinetic information to the mixture toxicity model is a point of refinement which could be added in the future (see Section 8.5), as indeed, at higher exposure levels most substances – including metals (Jain & Gauba, 2017; Fu & and Xi, 2020) – may have the capacity impact detoxification of additional substances. It is also possible that toxicodynamic synergism or potentiation could be relevant for the current substances, as they result in the same outcome (IQ loss) and possibly through similar or related pathways of toxicity (i.e., most known AOPs for DNT in the OECD AOP wiki include similar key events, however, it must be noted that none of these is specific for IQ loss). Nevertheless, there is no evidence for specific action at the same receptor(s), and synergism via simultaneous or sequential action via different receptors or mechanisms on the same key event during development remains theoretical.

8.4.2. Uncertainties in hazard data

The measurement of general intelligence via IQ testing is a perennially discussed topic (Kovacs & Conway, 2019; Silverman & Gilman, 2020; Gottfredson, 2000), and it is well known that the test can result in different results e.g., when the cultural background of the child being tested does not match that of the test (“dominant culture”) (reviewed by Weiss & Saklofske (2020)), which may not actually be reflective of IQ differences. In addition, although the events which lead to IQ loss can occur any time during the development of the brain, IQ can only be tested from around age 3 and is most reliable from age 6 (with increasing confidence with increasing age). For this case study, the HBM-G/TVs were derived from different types of IQ testing, with effects observed at different ages. This introduces additional uncertainty into the comparability of the IQ loss measured for each substance.

In addition to lack of comparability of IQ measurement and ages at which an effect was noted for each substance, the degree to which (and the type of) confounders which were accounted for in the chosen studies for the HBM-G/TV varies. Studies which accounted for the most confounders were chosen (lowest risk of bias), however, the comparability is nevertheless not exact. The diversity of the populations and locations of the populations is also not comparable, which can introduce minor uncertainty as to the level of genetic diversity which was covered.

8.4.3. Uncertainties in exposure

Individual variation in exposure can be quite high and, particularly in the case of heavy metals, can be highly influenced both by diet and by geographic location. In addition, since the measurements were performed in three different matrices, the comparability with type of exposure varies. Measurement of TRA in urine is possibly the most uncertain as far as long-term exposure is concerned, as, depending upon the type of urinary measurement (spot measurement or 24h) and length of follow-up (one time measurement or multiple) it may reflect either acute or more long-term exposure. Measurements of lead in blood and methylmercury in hair are more reflective of long-term exposures, however, when measurements of lead in urine were performed, the same uncertainties as reflected above for TRA exist. The use of conversion factors (for TAs or Pb) also introduces uncertainty to the exposure estimation.

8.5. Future perspectives

The case study presented here is a proof-of-concept for the use of HBM data in retrospective MRA and is not intended to illustrate risks from the substances analysed. Although this work represents an important “lower tier”, refinement of the results could allow an improved understanding of the potential for risk assessment based on measured residues in human matrices, and thus better contribute to risk management decision-making.

Examples of refinements which are currently being developed under the auspices of the PARC Real-life mixtures project include the use of PBK modelling to allow a more precise understanding of the level of each substance (metal) which would be expected to reach the brain of developing foetuses and children, refinement of HBM-GV/TVs to use full dose-response curves in the risk estimation (rather than the more uncertain single-point estimates), and the possible inclusion of indicative effect biomarkers in future HBM projects. Additionally, in PARC WP5 work continues on understanding the mechanisms whereby neural development is impacted by different types of substances, which might improve an understanding of the most sensitive timeframes/populations for different substances and a more refined/targeted risk assessment.

Finally, using MCRA it is possible to perform a sensitivity analysis related to the most important sources of uncertainty in order to determine which of these impacted the results. Since the current case study is a proof-of-concept, it is anticipated that such analysis will become even more relevant as additional refinements are included.

9. Mixture identification based on chemical co-exposure

Mixture components can be selected based on their hazard potential (hazard-driven criteria) as seen in the four case studies, or it can be based on co-exposure (exposure-driven criteria) (EFSA et al., 2021). HBM data can be used for this latter approach based on co-exposures. By studying the substances to which a group of individuals are commonly exposed using their HBM data, it is possible to define relevant mixtures for further analysis. Various statistical methods exist for identifying mixtures based on co-exposures, in the following sections one of them (the

SNMU) will be presented. These defined mixtures can be used as a basis for MRA that are based on “real-life” mixtures, provided that the hazard data for the selected substances are available.

9.1. Mixture identification from combined exposure

Case studies presented in Sections from 5 to 8 only consider chemical from common chemical families (pesticides, metals, PFAS). However, in real-life populations are exposed to various type of chemicals. Considering HBM data, the objective is to identify real-life mixture based on co-exposures across chemical families. Statistical methods can be applied to HBM datasets to identify these co-exposures. Applying the Sparse Non-negative Matrix Underapproximation (SNMU) approach to HBM data can identify single substances or mixtures that contribute most to the total exposure using a dimension reduction method (Gillis & Plemmons, 2013). More details about the SNMU approach and the associated clustering classification can be found in AD6.3 (PARC, 2023). This approach has been implemented in MCRA. The SNMU approached has been tested on HBM data (FLEHS 2) during HBM4EU (HBM4EU, 2022b).

The SNMU could be used to

- Extend the case studies: In the datasets involved in MRA case studies, co-exposures between substances prioritised in the case study and substances measured in the concerned individuals can be analysed.
- Identify common mixtures: datasets analysing similar substances could be grouped. The results of the SNMU from these datasets could provide insight of common mixtures to which European populations are exposed to. The latest findings could serve as the basis for an MRA.

The main challenges identified are the grouping of studies as there is a lot of heterogenicity among the datasets, and the selection of mixture of interest since it requires the expert judgment.

SNMU approach was applied on different sub-datasets of ESTEBAN (PARC, 2023). For example it was used on a sub-dataset of 427 children for which 28 metals and 23 pesticides where measured. Eight mixtures were identified showing potential co-exposure between the metals included in the DNT case study and some pesticides.

In year 4 we plan to apply the SNMU approach on the studies that have been already harmonised and uploaded in MCRA to extend case study and to identify common mixtures.

9.2. Estimating uncertainty in mixture identification statistical approach: application to SPECIMEN cohort.

9.2.1. Human biomonitoring studies

The SPECIMEn cohort, which is part of HBM4EU (Vitale et al., 2022; Ottenbros et al., 2023) contains information on pesticide biomarkers measured with a harmonised suspect screening approach in urine samples of 525 parent-child pairs in five EU countries and lifestyle factors (e.g., food preferences, time spent in different areas).

9.2.2. Aim of this case study

A commonly used unsupervised technique to identify real-life mixtures is sparse non-negative matrix underapproximation (SNMU) (Hippert et al., 2024). SNMU is a dimension reduction technique similar to Principal Component Analysis (PCA), but with positivity constraints and other convergence criteria included to improve estimation for non-negative data (Gillis & Plemmons, 2013). In essence, SNMU is a sparse bilinear matrix decomposition method, with positive scores and loadings. Despite the increased interpretation benefits of SNMU compared to PCA, there are some points in its application that need attention. First, stability (i.e., or uncertainty) is typically not assessed. Second, in view of the recursive nature of the algorithm, SNMU may be affected by the problem of deflation (Camacho et al., 2021).

This case study has a dual aim. First to address the above-mentioned concerns by developing a statistical framework for estimating uncertainty around the SNMU output and creating diagnostic tools for the deflation issue. Secondly, reanalyse the SPECIMEn dataset for real-life mixture identification and risk factor analysis of lifestyle variables associated with their presence. More specifically the current tasks under development are the following:

Uncertainty of identified real-life mixtures: Using bootstrap sampling the stability of the loadings for each identified mixture can be estimated and assessed. Using a statistical cut-off level, selection of the ones with enough confidence (not including zero) of being detected in the general population can be performed.

Number of identified real-life mixtures: Another aspect that needs assessment is the number of mixtures to extract. To this end, several criteria and expert opinion have been used (Mancini et al., 2021), but no optimal strategy exists. We are working on a pipeline to distinguish mixtures from noise through permutation testing. Briefly, the loadings within SNMU are permuted while preserving the SNMU scores, after which we compare the reconstruction error. The true model should have statistically lower error compared to the permuted models. Only a single component mixture is permuted at a time. The overall procedure is combined with bootstrapping, with the aim to robustly establish how many mixtures can be distinguished from noise. This work is still under development.

The deflation issue: Based on how the principal components (PCs) are extracted, algorithms used in dimension reduction techniques can be categorised into two categories. Algorithms estimate all PCs at once or sequentially one after the other (recursive). Recursive algorithms in sparse PCA have been identified to have a computational issue that might introduce bias into the results. This issue has been described in a series of papers (Camacho et al., 2020, 2021) and it refers to the bias introduced as the PCs are recursively computed in wide data sets where sparse loadings “can be outside the data row-space”. If this phenomenon occurs the impact is that artifacts of variance, not present in the original data are created during calculation of the residuals for the extraction of a component and thus the subsequent components are affected. There are no remedies to fix this when it takes place, but it can be diagnosed and taken into consideration during inference. Camacho et al. (2021) provides a pipeline with diagnostic tools. We aim to implement this pipeline for the SNMU framework and detect when and to what extent the deflation problem becomes an issue that needs attention.

By implementing the three above tasks further insights and interpretability of the SNMU output can be provided.

Life-style factors associated with real-life mixtures: After identifying the real-life mixtures within the SPECIMEn data set using the established framework, we will explore associations between life-style factors and estimated mixtures using a Random Forest (RF) approach. RF models were chosen because of the non-parametric nature and ability to capture interactions (Wright et al., 2016). A computational efficient and robust algorithm is the binomial RF (Zaim et al., 2019, 2020) which is intended to be used for this analysis using the SNMU-computed scores as single outcomes.

Currently this work is ongoing, and some initial results will be available by summer of 2025.

10. Biomarkers of effect and prioritised mixtures

A working group of 6 partners (VITO, ANSES, AU, NIPH, UU-IRAS, WR-BIOM, RECETOX) is studying the association between mixture exposure and biomarkers of effect or health effects in the HBM studies. The aim is to apply and evaluate different statistical multiple-pollutant methods (described in the Annex section 1):

- Single- & multiple-pollutant multiple regression models (spMLR & mpMLR)
- Weighted Quantile Sum regression (WQS)
- Quantile G-computation (QGcomp)
- Elastic net regression (ENET)
- Horseshoe regression (HS)
- Bayesian model averaging (BMA)
- Random forest (RF)
- Bayesian kernel machine regression (BKMR)

Different partners are working on a common set of health endpoints: hormones (4 case studies), blood pressure (2 case studies), immune effects (2 case studies), and Parkinson (1 case study). The research questions, the HBM data used, and the status of the different case studies is detailed in Table 12.

Table 12. Research questions studied and HBM data used by the different partners.

Partner	Research question	HBM Data	Status
Hormones			
VITO	Mediating effect of hormones in the association between mixture exposure and birth weight	Newborns FLEHS II & III, Flanders	Paper submitted for publication
AU-PH	Mixture exposure – thyroid & sex hormones	Pregnant women ACCEPT birth cohort, Greenland & FETOTOX Danish pregnant women	Analyses ongoing
RECETOX	PFAS mixture exposure – thyroid hormones	CELSPAC-FIREexpo case-control study, 18-35 years, Czech Republic	Analyses ongoing
NIPH	Mixture exposure – thyroid hormones	Children NEB II, 7-14 years, Norway	Analyses ongoing
Blood pressure			
VITO	Mixture exposure – blood pressure	Adolescents FLEHS III & IV, 14-15 years, Flanders	Final results
ANSES	Mixture exposure – blood pressure	Adults ESTEBAN, 18-73 years, France	Final results
Immune effects			
VITO	Mixture exposure – leucocyte & subtype counts	Adolescents FLEHS III & IV, 14-15 years, Flanders	Paper submitted for publication
NIPH	Mixture exposure – vaccine antibodies for MMR (Measles, Mumps, Rubella) and DTP (Diphtheria, Tetanus, Polio)	Children MoBa (NEB II & HELIX), 7-14 years, Norway	Analyses ongoing
Parkinson			
UU-IRAS	Mixture exposure – Parkinson	Nested case-control study within EPIC, 35-70 years, Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden, UK	Final results

10.1. Results mediating effect of hormones in the association between mixture exposure and birth weight (newborns FLEHS)

The potential mediating role of hormones in the association between prenatal chemical mixture exposure and birth weight was examined using data of 432 newborns from two Flemish birth cohorts (FLEHS II and III, 2008-2009 and 2013-2014, respectively). The common set of available and detectable exposure biomarkers and hormones analysed in cord plasma were: 6 metals/trace elements, 3 polychlorinated biphenyl (PCB) congeners, hexachlorobenzene (HCB), dichlorodiphenyldichloroethylene (DDE) and 2 perfluoroalkyl substances; and 3 thyroid, 3 reproductive and 2 metabolic hormones. Mixtures analyses were performed to assess each of the bilateral associations in the path exposures-hormones-birth weight, using mpMLR, BAS, and BKMR. Only PCB 180 was found to be associated with hormones as well as birth weight, so mediation analyses were restricted to single-exposure single-mediator models (using the R package “mediation”) and single-exposure multiple-mediator models (using high dimensional mediation analysis (HDMA) as implemented in the R package “hdmed”). Combining all exposures, we found an inverse association between PCB 180 and birth weight. PCB 180 was positively associated with sex hormone-binding globulin (SHBG) and negatively associated with leptin and insulin. Similarly,

thallium was positively associated with testosterone, estradiol, and SHBG, and negatively with insulin. Lead was positively associated with insulin. Higher free thyroxine (FT4), insulin, and leptin were associated with higher birth weight, whereas higher SHBG was associated with lower birth weight. Mediation analysis for PCB 180 indicated that a large part of the effect of this exposure on birth weight is mediated by FT4, SHBG, leptin, and insulin. A paper on this case study has been submitted in Environmental International and is currently under review.

10.2. Results mixture exposure - thyroid & sex hormones (ACCEPT & FETOTOX birth cohorts)

The associations between mixture exposure and thyroid and sex hormones are examined among pregnant women in two birth cohorts: the Greenlandic ACCEPT and the Danish FETOTOX. In the ACCEPT cohort exposure blood concentrations to PFASs, PCBs, organochlorine pesticides (OCPs), and metals were available together with thyroid hormone levels (Thyroid-stimulating hormone (TSH), total triiodothyronine (TT3), total thyroxine (TT4)). None of the multi-pollutant methods (WQS, QGcomp, BKMR, ENET, BMA, RF) provided strong evidence for associations, most likely due to a small study population (n=115). However, similar tendencies were seen with many of the six statistical methods. In the FETOTOX cohort, exposures to PFASs, PCBs, OCPs, and metals are available together with thyroid hormone levels (TSH, FT4, Thyroperoxidase (TPO)-antibodies) and sex hormones (Estradiol, Estron, Estrone sulfate, and Dihydrotestosterone). The analyses on the FETOTOX data are planned for spring/summer 2025.

10.3. Results mixture exposure - blood pressure (adolescents FLEHS)

Detailed results of this analysis are presented in the annex file. Except for the negative association between SumPCB and systolic blood pressure in mpMLR, none of the multi-pollutant methods provided evidence for associations between individual exposure biomarkers and blood pressure. QGcomp, however, indicated a significant overall mixture effect for both diastolic blood pressure and systolic blood pressure, pointing at a lower blood pressure in association with increased mixture exposure.

10.4. Results mixture exposure - blood pressure (adults ESTEBAN)

The association between metal exposure and blood pressure was examined in 1324 adults. Detailed results of this analysis are presented in the annex file. No strong evidence of associations was identified. The different models applied here did not provide strong evidence for associations between metal exposure biomarkers and blood pressure. Nevertheless, a positive association was found with WQS on diastolic blood pressure. Some refinements could be carried out such as raising the number of iterations for BKMR models, correcting for the age and sex interaction before applying the models or adding more cofounders related to BP (such as BMI, smoking or individual dietary salt intakes). Due to the lack of overall evidence of an association between metals and BP this associations will not be further explored. A similar work was conducted on the same study (ESTEBAN) considering metals (36 chemicals) and PCBs (53 chemicals). The study population was smaller (n= 485 adults). Due to high correlations, PCBs were studied as a sum. The conclusions for this subpopulation were similar to the ones presented in the annex.

10.5. Results mixture exposure - leukocytes (adolescents FLEHS)

The association between mixture exposure and white blood cells was examined in 980 adolescents (13-16 years old) from the Flemish Environment and Health Studies 2012-2020 (FLEHS III and IV). We modelled counts of total leukocytes, counts and proportions of leukocyte subtypes (neutrophils, lymphocytes, monocytes, eosinophils, basophils), and the neutrophil-to-lymphocyte ratio. We used the same exposure biomarkers (with the same treatment of censored values, blood lipid correction, and transformation) as in the blood pressure case study, i.e., 5 metals, 3 chlorinated pesticides, and 6 PCBs measured in blood. We also applied the same statistical models as in the blood pressure case study, correcting for the following covariates: campaign (FLEHS III versus IV), age (in months), sex, highest educational level of the household (International Standard Classification of Education (ISCED)), and body mass index (BMI). The estimation of the overall mixture effect was not meaningful in this case study because observed associations were not always in the same direction. Therefore, we did not report results

from weighted index model (WQS and QGcomp) which are appropriate for estimating the overall mixture effect but not for the identification of relevant exposure biomarkers. We also did not report results of the HS because of the similarity of this method with ENET.

Results of spMLR, mpMLR, ENET, and BMA suggested immunosuppressive effects of PCBs and some metals, but only a few of these associations were picked up by BKMR. Although this discrepancy might be explained by the existence of nonlinear exposure-response associations or interactions between exposure biomarkers, BKMR plots did not show strong evidence for nonlinearity or interaction effects. Our study provides epidemiological evidence that exposure to metals and PCBs may have adverse effects on the immune system at concentrations detected in a general population of adolescents. A limitation is however the cross-sectional design of the study, so the possibility of reverse causation cannot be ruled out. A paper on this case study has been submitted in *Environmental International* and is currently under review.

10.6. Results mixture exposure - Parkinson disease (EPIC)

WQS regression and QGcomp were used to estimate the overall mixture effect of metals on Parkinson disease (PD) case-control status. Penalised regression (ENET & HS) and Random Forest (RF) models were used to identify potentially important mixture components. The data used was from a case-control study that was nested within the large EPIC cohort and included 362 PD cases and 362 controls that were matched on age, sex, and recruitment center. Metals were measured in blood samples obtained at entry into the cohort and included As, Ca, Cd, Cu, Fe, Hg, Mg, Mn, Pb, Se, and Zn. The results from the WQS models did not suggest a strong association between metal exposure and case-control status, with estimated Odds Ratios (OR) [95% confidence interval (CI)] of 0.98 [0.91, 1.07] for exposure to the mixture in the full population and 1.00 [0.58, 1.73] in smokers. The QGcomp model resulted in a similar, but more precise, estimate for the full population (OR [(95%CI) = 0.97 [0.94, 1.01]], but a much stronger negative estimate in smokers (0.79 [0.67, 0.92]). In that last model, Pb appeared to contribute most to the overall mixture effect. None of the individual metals were found to be significantly associated with case-control status using the penalised regression models, although in the HS regression models Pb consistently showed the strongest effects both in the full population (OR [95%CI] = 0.99 [0.95, 1.04]) and in smokers (0.73 [0.31, 1.03]). In RF models, which allow for interactions and non-linear exposure-response relations, Pb was found to be the second most important exposure, the first being Cd which also showed an inverse relation with case-control status, albeit only at the extreme low range of exposures.

10.7. Pros and cons of the statistical methods

- This section summarises the pro and cons of the different statistical multiple-pollutant methods applied to study the association between the mixture biomarkers of exposure and biomarkers of health effects. spMLR

Does not account for potential confounding by co-exposures in the mixture.

- mpMLR
 - Can in principle avoid confounding by co-exposures, but only when there is no unmeasured confounding. In case of unmeasured confounding of some individual substances of the mixture, the inclusion of correlated exposures in one regression model may even result in amplification of the confounding bias (Weisskopf et al., 2018). This issue may also occur in other multi-pollutant methods.
 - Model estimates may be imprecise in small datasets with many (highly) correlated exposures.
- Weighted index methods (WQS and QGcomp)
 - Provide an estimate of the overall mixture effect. However, when the mixture that is studied is rather heterogeneous (as in the blood pressure and leukocyte case studies), estimating an overall mixture effect may not be useful because the directions of associations between health outcome and individual substances in the mixture may not always be the same. The algorithm implemented in the WQS package allows for only a single mixture and requires the effects of components within a mixture to have the same sign.

- These methods may not be the best when the goal is to identify mixture components (substances) that seem important or for estimation of substance-specific effects. The estimated weights provide some indication of the relative importance for individual substances, although this is really only useful when the precision of these weights can be taken into account (e.g., using bootstrapping).
 - QGcomp estimates the overall mixture effect using a marginal structural model, which may improve causal interpretation of the estimated effect. Note however, that this requires several causal assumptions to hold that in most cases cannot be verified.
- ENET
- A penalised regression method that reduces overfitting and allows for variable selection.
 - Runs relatively quickly, even on large datasets with many exposures and can be used even when the number of variables in the model exceeds the number of observations.
 - Final coefficient estimates for the effects of selected variables are likely to be biased downwards, although attempts have been made to overcome this issue (e.g., the debiased LASSO, (Vazquez & Nan, 2025)).
 - Provides no direct information regarding the precision of effect estimates. The stability of the model can be investigated using the stability selection algorithm.
- HS
- A Bayesian penalisation method that uses a somewhat more complex penalty (horseshoe regression), which reduces coefficient bias and provides 95% credible intervals for estimated coefficients that can be used to evaluate uncertainty in estimated effects under the assumed model.
 - Results from Bayesian regularisation models cannot be used directly for variable selection, although this is possible with some additional steps. One approach would be the selection of variables for which the 95% credible interval does not include zero.
- BMA
- The R package BAS provides an efficient Markov chain Monte Carlo (MCMC) algorithm to sample the model space in case a high number of exposures makes the complete enumeration of all possible models unfeasible. Provides the ability to evaluate the impact of different priors on the model space and model coefficients.
 - Provides posterior model probabilities for potential model structures, posterior inclusion probabilities (PIPs) for individual exposures, and model-averaged estimates of exposure effects.
- RF
- Allows for non-linear effects and complex interactions between exposures, but this also makes the results hard to summarise.
 - Variable importance (VIMP) scores can be used to identify important exposures, although these tend to suffer from the same problems as the weights estimated by WQS.
 - There is no pre-defined threshold for VIMPs that can be used for variable selection, but the informal “elbow method” can be used, i.e., plotting sorted importance values and looking for distinct breaks. In addition, one can use the Boruta package to perform an iterative top-down search for all relevant features by comparing original importance estimates with those for permuted variables.
 - Partial dependence plots can be used to show the dependence between an outcome and one or two exposure values after marginalising over the values of all other variables in the model but are less useful when effects are heterogeneous between subjects due to interactions with other variables. In this case dependence can be visualised using Shapley values, which also display the variance on the y-axis. A disadvantage is that Shapley values are computationally intensive to calculate.
- BKMR
- The same as for RF, the main advantage of BKMR is that it allows for non-linear effects and interactions between exposures, but this comes with the downside that results are less easy to interpret/summarise.
 - Provides an estimate of the overall mixture effect.

- The estimation process can take a very long time and becomes challenging for a dataset with more than a few 1,000 observations.
- Requires critical checking of model convergence. The default implementation (in the R package `bkmr`) allows for only a rudimentary check, so we recommend running at least 4 different Markov chains (using the `bkmrhat` package to allow a more complete check), but this implies proportionally longer running times.
- The use of one of the two exact methods for the estimation of posterior summaries provides unbiased estimates if the BKMR model is correctly specified and if the model has converged, but the methods take a long time to run. The approximate method often seems to yield strongly biased estimates (e.g., significant effect estimates whereas observed PIP values were very low).
- In the absence of strong nonlinear or interaction effects, one would expect that BKMR results are similar to those from linear models, but due to the larger flexibility, i.e., higher number of degrees of freedom, BKMR might not pick up associations identified by other methods (as we observed in our leukocyte study).

11. Regulatory relevance of PARC 6.2.3 results

One of the main results provided by the PARC project Real-life mixtures is the identification of chemicals that contributes most to the risk related to real-life mixtures called risk drivers. At the stage of the project, it was only possible for identify the chemicals observed in HBM studies driving the risk.

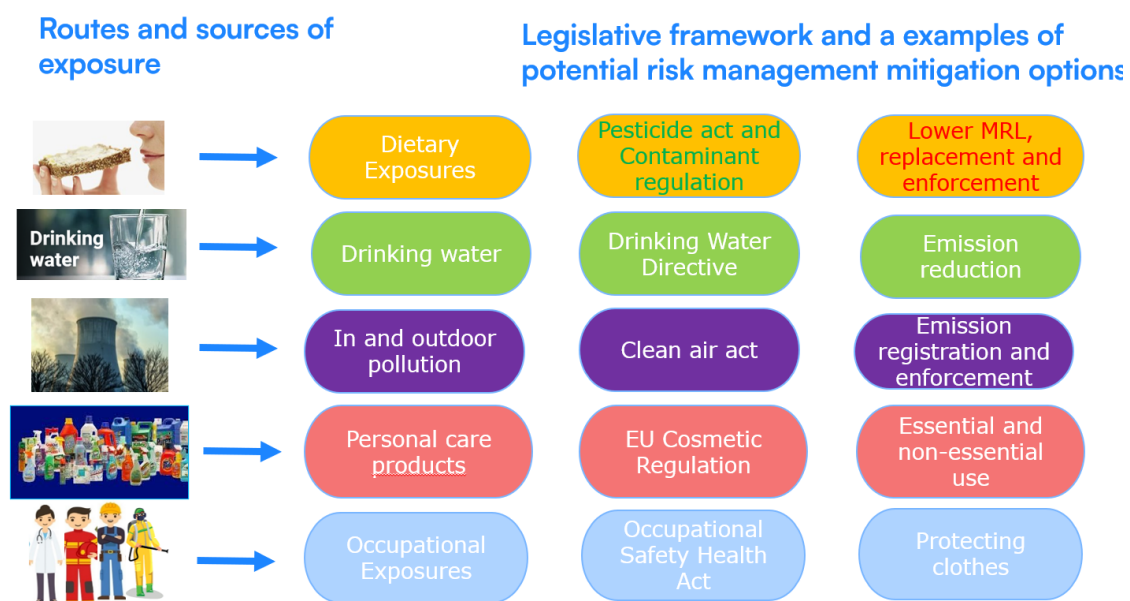


Figure 18. Regulations that might help risk managers to reduce the risk based on risk mitigation measures

Figure 18. shows that risk managers working in different regulatory silos are working according to a legislative framework. DG SANTE can set lower maximum limits for food contaminants after discussion in their working groups.

For risk mitigation measures, modelled exposure estimates are needed. In a later stage it is foreseen that the sources of exposure will also be enriched by results from the PARC projects P6.2.1.a Source-to-dose and P6.2.1b aggregated exposure. In the meantime, discussions were initiated by working in synergy with EFSA. EFSA offered help to organise food consumption and residue monitoring data for modelling dietary intake. Once this is achieved, it might offer options for risk mitigations, Maximum limits are laid down in R 1881/2006. Regulation R 2023/915 offers possibilities to lower limits or to set new limits for example for heavy metals in food. DG SANTE's Committee DG SANTE Working Group on Industrial and Environmental Contaminants.

DG SANTE and PARC case study leaders discussed options to perform future scenario analyses with in- and exclusion of samples not compliant to the current legal limits. If an impact might be concluded from these scenario

analyses new MLs might be considered or enforcement activities on food items exceeding existing ML might be strengthened.

The next steps to be taken in the years 2026-2029 is then to further discuss the comparison between modelled intake using external exposure data and model compared to observed concentrations based on HBM studies with the European Agencies and European Regulators. The focus of the discussion with regulators will then be on regulatory actions that can be taken to minimise observed high exposure to mixtures. This discussion was initiated at the PARC – DG SANTE meeting held on 29th May 2024 and 18th January 2025. Further improvements on the proof-of-concept and uncertainties by cooperation with European Agencies responsible for European risk assessment is recommended. The comparison between dietary intake and observed intake may help to understand the relevance of the dietary route of exposure.

According to the Occupational Safety Health exposure limits can be set to avoid exposure to harmful substances at working places. Under the REACH regulation, substance of very high concern can be restricted by understanding and deciding on essential and non-essential uses.

The harmful effect of a combination of Substances of Very High Concern (SVHC) may be greater than the effect of each individual substance. Very limited consideration is given to such combination effects when issuing permits. It is also not established.

Risk mitigation for SVHC is also addressed in restriction measures for example for PFAS. ECHA and authorities from Denmark, Germany, the Netherlands, Norway and Sweden have released a progress update on the process to restrict per- and polyfluoroalkyl substances (PFAS) (ECHA, 2024)

In the context of the process to restrict PFAS exposure, dossier submitters submit their dossiers to ECHA, and ECHA's scientific committees for Risk Assessment (RAC) and for Socio-Economic Analysis (SEAC) continue to consider more potential restrictions in uses. This process helps the Dossier Submitters to progressively update and improve the information on PFAS.

Many substances of very high concern are already in use. Although their uses can be more restricted, many of these chemicals occur for a longer time in the environment due to their persistency. These chemicals enter the food chain and concentration limits for these chemicals are set in food and drinking water.

12. Conclusion and next steps

This report outlines the progress made in applying the MRA strategy developed in this project in a first-tier risk assessment for the case studies prioritised by the PARC Governing Board members. This report focuses on procedures, scientific principles and development of operational tools to use HBM data for MRA more than formal risk assessments. We have performed MRA in a harmonised way to various European HBM datasets in applying several risk metrics, all based on dose addition approach recommended by EFSA. Harmonised protocols were followed by the HBM study owners and the MCRA software from the PARC model network was used by the HBM study owners to generate harmonised results at the European level. The results underline that, using the proposed methodologies and data, potential concerns regarding exposure to multiple chemicals were estimated and substances that contribute the most to those potential concerns are identified. These results were presented to European Agencies and to risk managers of the European Commission. This contributed to awareness that MRA using HBM data is feasible in Member States. Further discussion is needed on risk management options on how to reduce the exposure to identified chemicals contributing mostly to the concern. Uncertainties in exposure assessment and in hazard assessment were identified for each case studies. Possibilities to reduce these uncertainties will be discussed with European Agencies and/or PARC stakeholders having access to more refined data. This will be part of the second half of 2025 and early 2026 in organising meetings as well as to reinforce collaborations with EU agencies and stakeholders. More information will become available in the scientific publications that are expected from this work during the years 2025 and 2026. Several improvements are ambitioned for the next years of the Real-life mixtures project of PARC, such as enlarging the mixtures and refining the MRA strategy.

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Partnership for the Assessment of Risks from Chemicals

Supplement Deliverable D6.3

WP 6 – T6.2



Partnership
FOR THE
Assessment
OF
Risks
FROM
Chemicals



Co-funded by
the European Union

This partnership has received funding from the European Union's Horizon Europe research and innovation programme under Grant Agreement No 101057014.

1. Biomarkers of effects and prioritised mixtures

1.1. Description of the statistical methods

Using single-pollutant linear regression models to evaluate the health effects of chemical agents that occur in mixtures is problematic because it ignores potential confounding by other substances in the mixture and does not allow for identification of interactions between substances or evaluation of an overall mixture effect. With a small set of exposures or a large dataset, the classical multiple linear regression model can be used to estimate both main effects and interactions in many cases, although estimates may be too imprecise to be useful when there are strong correlations between exposures.

Epidemiological analyses of health effects associated with mixture exposure can address different research questions:

1. Mixture identification, i.e., which exposures tend to occur in combination?

This is usually done as a first, exploratory analysis and involves assessing the correlation between the different substances of the mixture, e.g., using Pearson's or Spearman's correlations for continuous measures. When there are (relatively) few observations and in the presence of highly correlated exposures, there may be insufficient power to estimate effects of single mixture components (research question #2). In that case, it may be better to select one of the components as representing all (correlated) exposures in a cluster, to use the sum of the components, or to extract the first component score from a principal components analysis (PCA) on the set of correlated exposures that make up the cluster. These so-called mixture- or cluster-prototypes can then be used as surrogates for the individual mixture components in further downstream analyses.

2. Which of the mixture components are important and what effects do they have?

The identification of exposures that are important risk factors, either alone or in combination, is often the focus of mixture-health effect analyses. Because there is a trade-off between model complexity and both interpretability and sensitivity, we propose to base inference on results from a range of models (multi-model inference). These models differ with respect to the type of effects that can be estimated (linear vs. non-linear, either including or excluding interactions), but also in the way they allow critical assessment of model quality and uncertainty. We distinguish between 3 types of methods:

1. Penalized (linear) regression models, e.g., elastic net (ENET) and horseshoe regression (HS).
2. Bayesian model averaging that allows explicit evaluation of posterior probabilities for different (linear) model structures, as implemented in the R package BAS.
3. Approaches that perform non-parametric estimation of multi-dimensional non-linear interaction surfaces: Random Forest (RF) and Bayesian Kernel Machine Regression (BKMR).

3. What is the overall effect of the mixture?

When different substances share a mode of action or have similar health effects, it might be of interest to estimate the total mixture effect. This can be done by:

4. One of the weighted index methods such as weighted quantile sum regression (WQS) and quantile G-computation (QGcomp)
5. BKMR

More details on the above methods are given in additional deliverable AD6.5 (PARC, 2023) and in [Report T4.1.4 - Statistical Analysis Plan \(SAP\) for T4.1](#). We also provide example scripts for each of the above methods, specifically written for PARC, available in the P6.2.3 SharePoint.

1.2. Result blood pressure FLEHS

Data

- N=988 adolescents from the Flemish Environment and Health study (FLEHS), campaigns III (2012–2015) & IV (2016-2020): 14-15 years, Flanders, Belgium
- Outcomes: diastolic & systolic blood pressure (DBP & SBP), mean of 5 consecutive measurements
- Exposure biomarkers: Selection of those detected in at least 70% of the samples of each campaign:

Metals in blood	
Cadmium	Cd
Copper	Cu
Manganese	Mn
Lead	Pb
Thallium	Tl
Chlorinated pesticides in serum	
Oxychlorodane	OXC
Dichloro-diphenyl-dichloroethylene	DDE
Trans-nonachlor	TN
Polychlorinated biphenyls in serum	
Polychlorinated biphenyl 118	PCB118
Polychlorinated biphenyl 138	PCB138
Polychlorinated biphenyl 153	PCB153
Polychlorinated biphenyl 170	PCB170
Polychlorinated biphenyl 180	PCB180
Polychlorinated biphenyl 187	PCB187

- Values below LOD/LOQ were imputed by random single imputation using a censored lognormal distribution.
- Lipid-soluble serum biomarkers adjusted for total blood lipid concentration (Phillips et al., 1989; Bernert et al., 2007)(Phillips et al. 1989, Bernert et al. 2007).
- LN-transformed, centered (subtracting the mean) and scaled (divided by the standard deviation).

Methods

- Covariates came from questionnaires (age (in months), sex, highest educational level of the household (ISCED), physical activity, smoking, alcohol consumption) or were measured (BMI). Missing values (<1.5%) were imputed (per campaign) using R package mice.
- Models
 - Single- & multiple-pollutant multiple linear regression (spMLR & mpMLR)
 - Weighted quantile sum regression (WQS)
 - Quantile G-computation (QGcomp)
 - Elastic net (ENET)
 - Bayesian model averaging (BMA)
 - Random forest (RF)
 - Bayesian kernel machine regression (BKMR)

Results

- Due to the high correlations between PCB 138, 153, 170, 180 & 187 (≥ 0.89 , Figure 1), their sum ("SumPCB") was used in further analyses (except in BKMR with hierarchical variable selection). This aligns with the chemical properties of PCBs, as these 5 highly correlated PCBs fall within the category of non-dioxin-like PCBs, while PCB 118 is considered dioxin-like (Serdar et al., 2014)

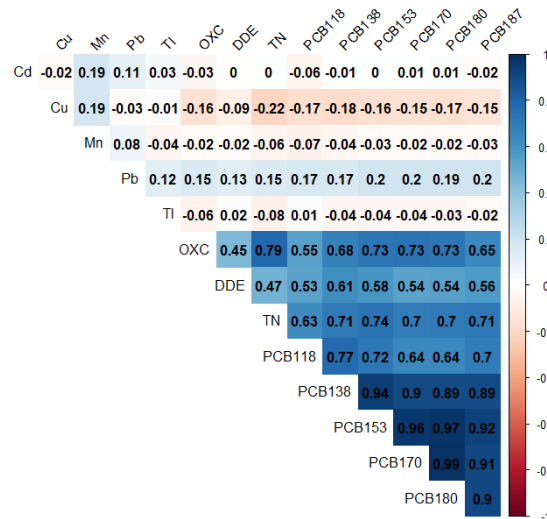


Figure 1 Spearman correlations between exposure biomarkers

- Results of **spMLR** models showed a negative association (P -value < 0.05) of OXC, TN, and SumPCB with DBP, but P -values of these exposure biomarkers were >0.1 in the **mpMLR** model (Table 5). A negative association between SumPCB and SBP was observed in both spMLR and mpMLR.

Table 1 Associations between exposure biomarkers and DBP and SBP, estimated by spMLR and mpMLR, with estimates representing the change in blood pressure (BP) (mm Hg) with 95% confidence interval (CI) per interquartile fold change (IQFc) in exposure biomarker.

Exposure biomarker	Estimate (95% CI)			
	DBP		SBP	
	spMLR	mpMLR	spMLR	mpMLR
Cd	-0.44 (-0.94; 0.07)*	-0.38 (-0.91; 0.15)	-0.45 (-1.08; 0.19)	-0.37 (-1.03; 0.29)
Cu	0.05 (-0.53; 0.62)	0.04 (-0.54; 0.63)	-0.04 (-0.75; 0.68)	-0.06 (-0.79; 0.66)
Mn	-0.54 (-1.13; 0.05)*	-0.49 (-1.10; 0.12)	-0.48 (-1.21; 0.26)	-0.38 (-1.14; 0.39)
Pb	0.22 (-0.45; 0.89)	0.39 (-0.30; 1.08)	-0.07 (-0.90; 0.76)	0.09 (-0.77; 0.95)
Tl	0.24 (-0.34; 0.83)	0.14 (-0.46; 0.73)	0.32 (-0.42; 1.05)	0.22 (-0.52; 0.96)
OXC	-0.68 (-1.29; -0.07)**	-0.11 (-1.02; 0.80)	-0.56 (-1.33; 0.20)	0.15 (-0.99; 1.28)
DDE	-0.29 (-0.89; 0.30)	0.06 (-0.64; 0.76)	0.12 (-0.63; 0.86)	0.70 (-0.17; 1.58)
TN	-0.79 (-1.42; -0.15)**	-0.43 (-1.40; 0.53)	-0.78 (-1.57; 0.01)*	-0.48 (-1.68; 0.73)
PCB118	-0.25 (-0.85; 0.36)	0.40 (-0.46; 1.26)	-0.35 (-1.10; 0.41)	0.31 (-0.76; 1.38)
SumPCB	-0.94 (-1.70; -0.18)**	-0.97 (-2.22; 0.28)	-1.11 (-2.06; -0.17)**	-1.62 (-3.18; -0.06)**

* P -value<0.1, ** P -value<0.05, *** P -value <0.01.

- For DBP, results of **WQS** (categorizing exposure into deciles) using negative repeated holdout validation (deriving weights from mixture effect parameters that are negative) showed highest weights for Mn and Cd (Figure 2, left panel), with respectively 82% and 79% of the repetitions having a weight above the threshold of 1/N exposure biomarkers (=0.1, the red line in Figure 2). For SBP, highest weights were observed for SumPCB and Cu (Figure 2, right panel), with respectively 74% and 68% of the repetitions having a weight above the threshold of 0.1. The overall mixture estimate for a 1 decile increase in all exposure biomarkers was not significant for DBP (-0.38 mm Hg, 95% CI: -0.77, 0.01), nor for SBP (-0.42 mm Hg, 95% CI: -0.88, 0.05). Results from the positive repeated holdout validation model (deriving weights from mixture effect parameters that are positive) indicated that there were no positive associations between exposure biomarkers and BP.

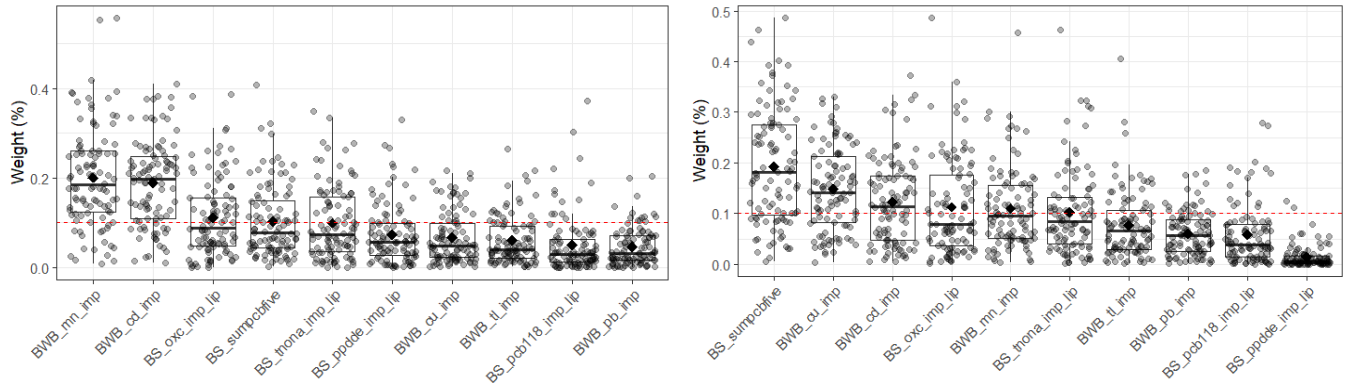


Figure 2 Weights estimated by WQS using negative repeated holdout validation for the association between exposure biomarkers (categorized into deciles) and DBP (left panel) and SBP (right panel). The horizontal redline represents the threshold of 1/N exposure biomarkers (=0.1).

- Consistent with WQS, **QGcomp** estimated negative weights for Mn and Cd in association with DBP, and for SumPCB in association with SBP. The highest weight for SBP was however a positive weight for DDE. The overall mixture estimate (for a 1 decile increase in all exposure biomarkers) was significantly negative for both DBP (-0.48 mm HG, 95% bootstrap CI: -0.94, -0.02) and SBP (-0.60 mm HG, 95% bootstrap CI: -1.18, -0.01).

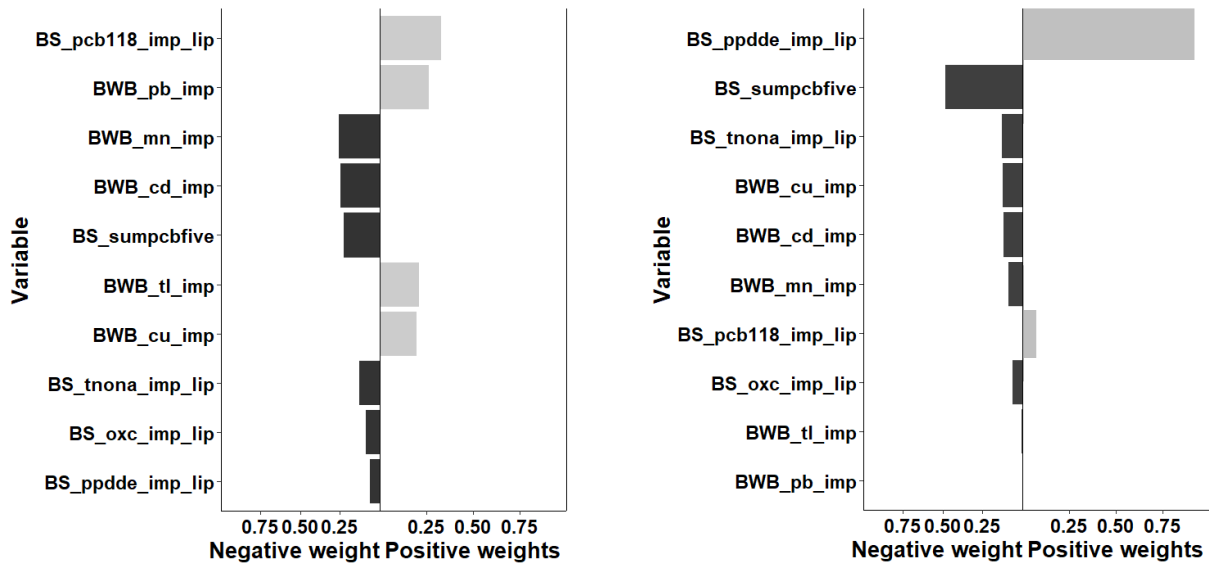


Figure 3 Weights estimated by QGcomp for the association between exposure biomarkers (categorized into deciles) and DBP (left panel) and SBP (right panel).

- Stability selection for **ENET** showed the highest selection frequency for SumPCB in association with SBP, but the threshold of 0.8 was not met, so there was no strong evidence for an association between BP and any of the exposure biomarkers (Figure 4).

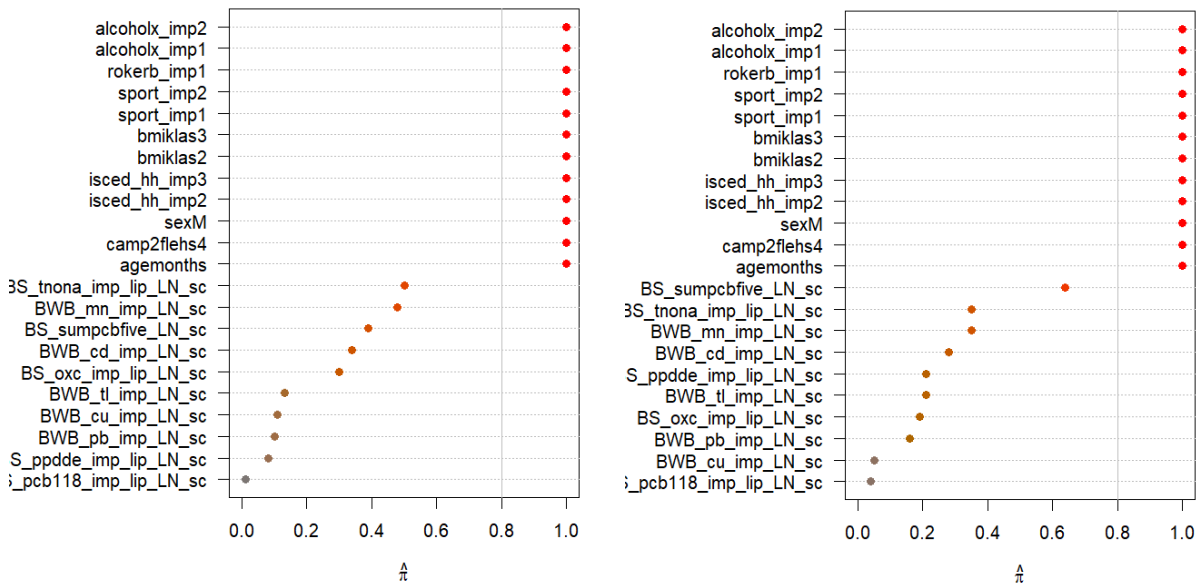


Figure 4 Stability selection for ENET models for DBP (left panel) and SBP (right panel): selection frequencies for the different exposure biomarkers, using a threshold of 0.8 for the selection frequency and targeting a per-family error rate of 0.5.

- Marginal posterior inclusion probabilities (PIPs) estimated by **BMA** were highest for SumPCB but were low (a PIP threshold of 0.5 or higher is typically used as a cut-off for variable selection), indicating no evidence for an association between exposure biomarkers and BP (Table 2).

Table 2 Associations between exposure biomarkers and DBP and SBP, estimated by BMA, with estimates representing the change in BP (mm Hg) with 95% credible interval (95% CrI) per IQFc in exposure biomarker.

Exposure biomarker	DBP		SBP	
	PIP	Estimate (95% CI)	PIP	Estimate (95% CI)
Cd	0.08	-0.03 (-0.36; 0.00)	0.03	-0.01 (0.00; 0.00)
Cu	0.02	0.00 (0.00; 0.00)	0.01	0.00 (0.00; 0.00)
Mn	0.09	-0.05 (-0.52; 0.00)	0.03	-0.01 (0.00; 0.00)
Pb	0.03	0.01 (0.00; 0.00)	0.01	0.00 (0.00; 0.00)
Tl	0.03	0.01 (0.00; 0.00)	0.02	0.00 (0.00; 0.00)
OXC	0.10	-0.06 (-0.66; 0.00)	0.03	-0.01 (0.00; 0.00)
DDE	0.03	0.00 (0.00; 0.00)	0.03	0.02 (0.00; 0.00)
TN	0.16	-0.12 (-0.93; 0.00)	0.07	-0.05 (-0.58; 0.00)
PCB118	0.03	0.00 (0.00; 0.00)	0.02	0.00 (0.00; 0.00)
SumPCB	0.17	-0.15 (-1.14; 0.00)	0.15	-0.17 (-1.40; 0.00)

- Variable importance (VIMP) plots of **RF** models indicated the importance of some covariates (age for DBP and BMI, FLEHS campaign, and gender for SBP), but did not provide strong evidence for important exposure biomarkers (Figure 5). A comparison of the VIMP of pairs of variables (“paired VIMP”) with the sum of individual VIMPs (“additive VIMP”) did not show evidence for strong interaction effects between exposure biomarkers (results not shown). The Boruta algorithm confirmed the importance of the variable age for DBP, and the variables BMI, campaign, and gender for SBP (“confirmed” features), whereas TN was classified as a “tentative” feature for DBP, and Cu and PCB118 were classified as “tentative” features for SBP. The partial dependence plots, showing estimated marginal effects for these exposure biomarkers, are presented in the upper panel of Figure 6, and the dependence plots using Shapley values in the lower panel. The Shapley value of a predictor for an observation explains the deviation of the prediction for that observation from the average prediction, due to the predictor.

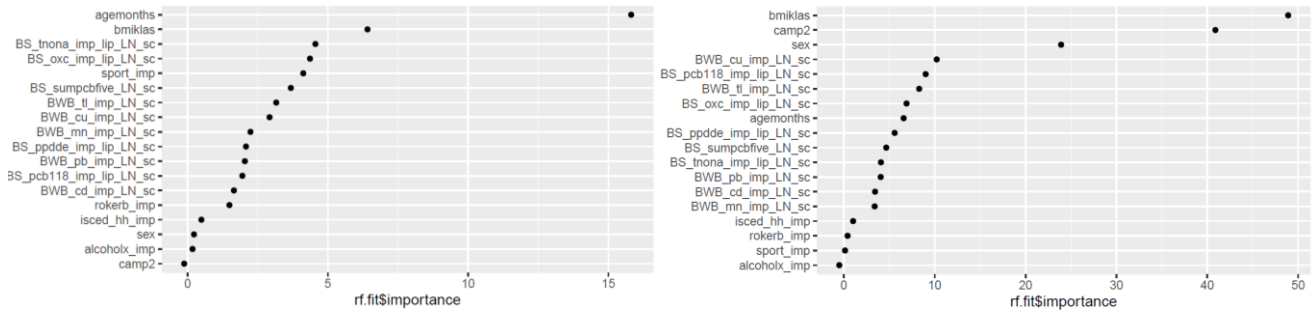


Figure 5 Variable importances for the association with DBP (left panel) and SBP (right panel) estimated by RF.

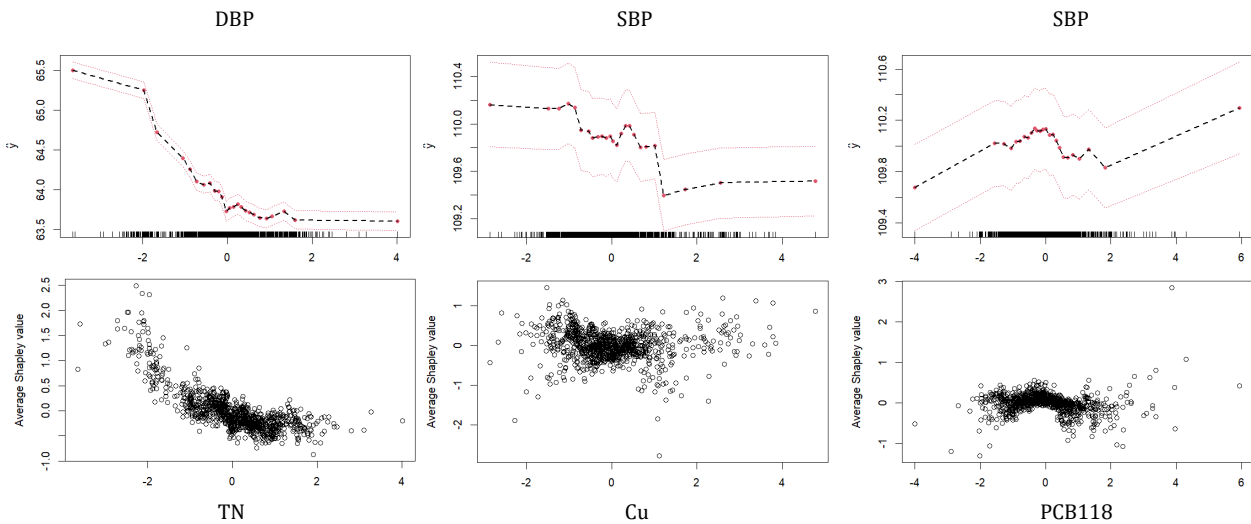


Figure 6 Partial dependence plots (upper panel) and dependence plots using Shapley values (lower panel) for the association between BP and exposure markers that were classified as “tentative” by the Boruta algorithm.

➤ In the **BKMR with component-wise variable selection** (variable selection on the individual exposures), PIPs were very low (<0.06; Table 3). For all exposure biomarkers, bivariate exposure-response plots showed parallel curves when the second exposure was fixed at different quantiles, indicating no important interactions (Figure 7 for DBP, results not shown for SBP). Figure 7 shows effect estimates for an IQFc in individual exposures (fixing other exposures at different percentiles) and Figure 8 presents the overall mixture effect, obtained by the approximate estimation method (upper panel in both figures) and the exact estimation methods (lower panels). A comparison of these posterior summaries indicated that the approximate method yields strongly biased estimates (effect estimates further away from zero), resulting in a significant overall effect for DBP estimated by the approximate method but not by the exact method.

Table 3 PIPs from BKMR models with component-wise variable selection.

Exposure biomarker	DBP	SBP
Cd	0.003	0.005
Cu	0.002	0.000
Mn	0.004	0.000
Pb	0.004	0.012
Tl	0.000	0.000
OXC	0.018	0.007
DDE	0.004	0.006
TN	0.059	0.015
PCB118	0.001	0.001
SumPCB	0.021	0.048

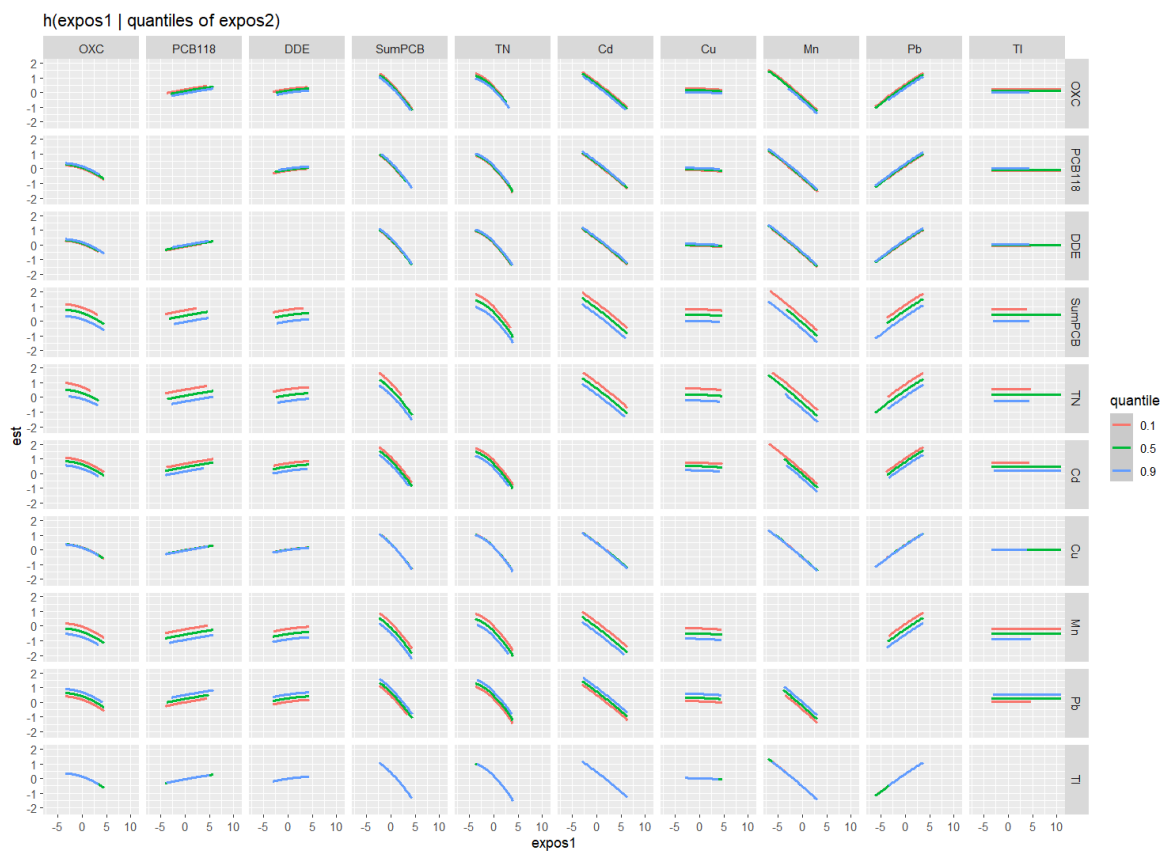


Figure 7 Predictor-response function for exposure1 (column header), fixing exposure2 (row header) at P10, P50, P90 (and fixing the remaining exposures at P50), estimated by BKMR with component-wise variable selection for DBP.

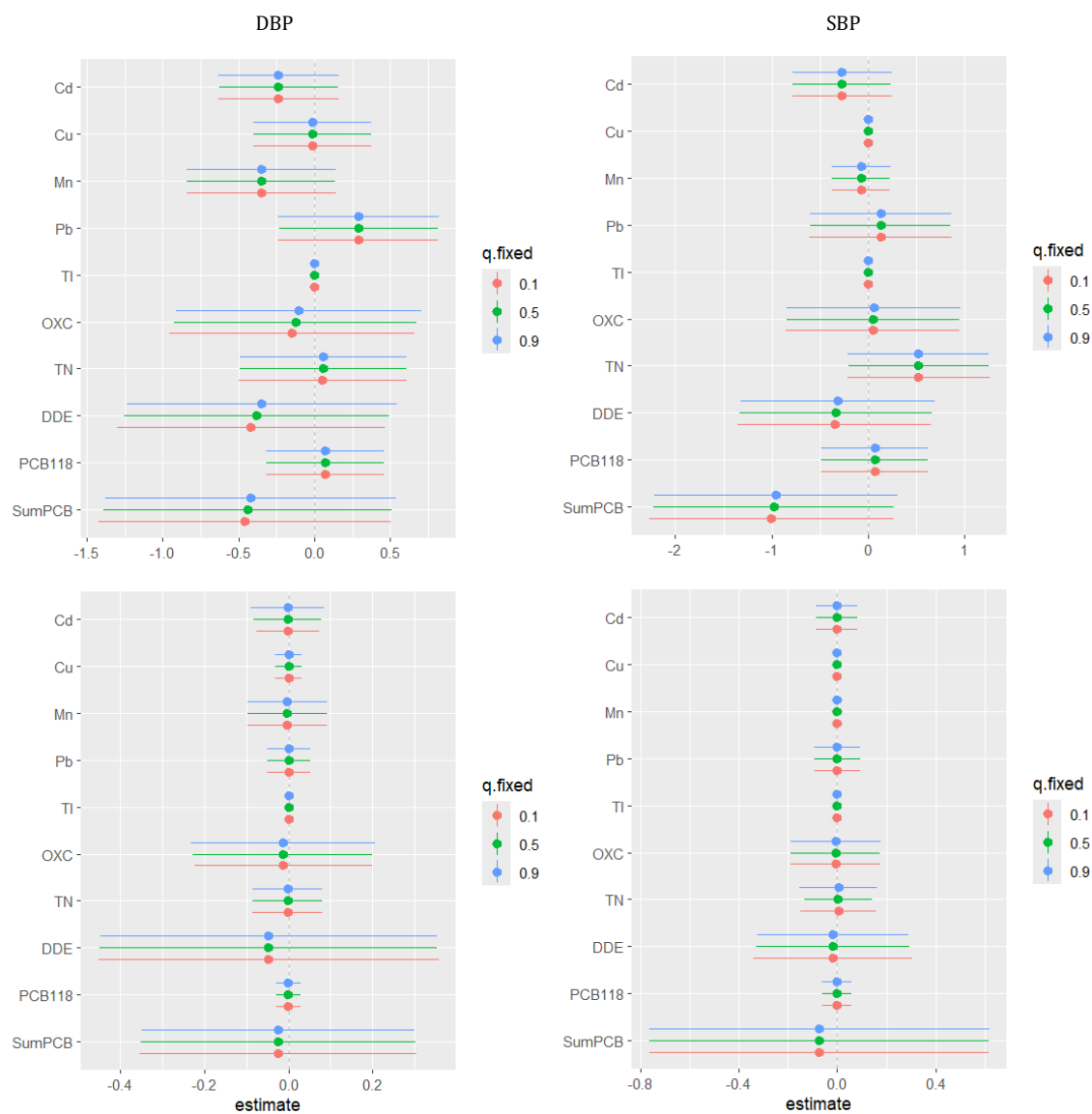


Figure 8 The estimate for an IQFc of the exposure (with 95% CrI), fixing other exposures at P10, P50, P90, estimated by BKMR with component-wise variable selection for DBP (left panel) and SBP (right panel), using the approximate (upper panel) and exact (lower panel) estimation method.

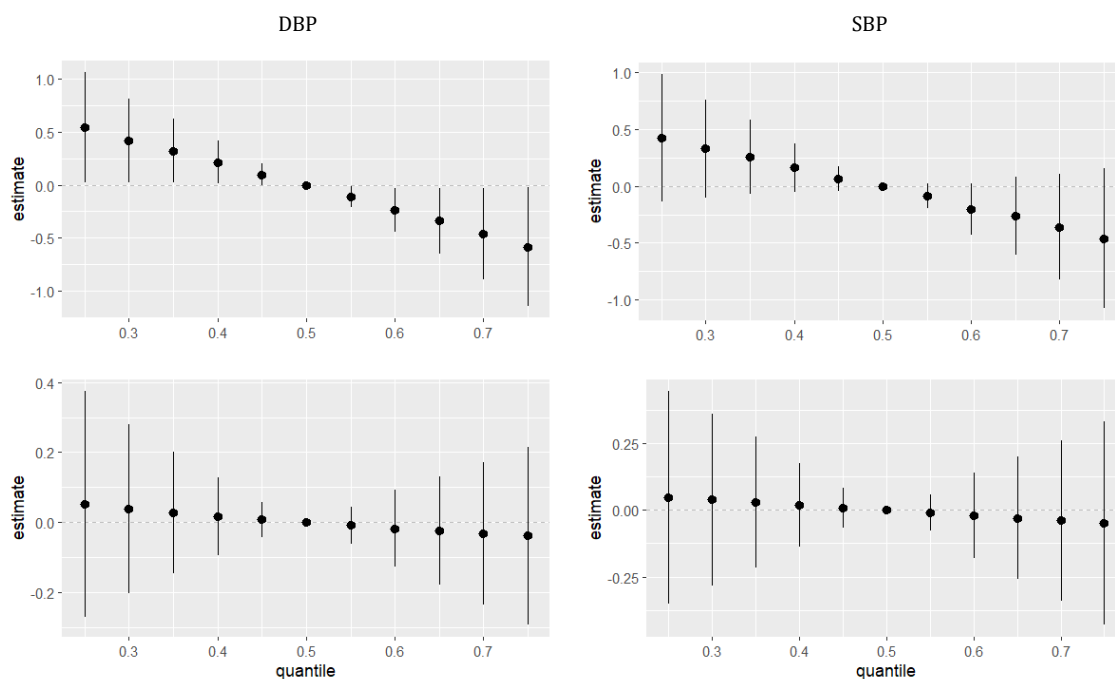


Figure 9 The overall effect of the exposure biomarkers at a particular percentile as compared to when all of them are at their median value, estimated by BKMR with component-wise variable selection for DBP (left panel) and SBP (right panel), using the approximate (upper panel) and exact (lower panel) estimation method.

- In the **BKMR with hierarchical variable selection**, highly correlated exposure biomarkers were grouped, i.e. OXC and DDE in one group, the 6 PCBs in another group, and the remaining 6 biomarkers were treated as 6 additional groups (Table 4). Highest group PIPs were observed for the group of PCBs but these PIPs were low (0.170 for DBP and 0.126 for SBP). PCB170 appeared to be the most important exposure in the DPB analysis (conditional PIP = 0.797), whereas in the SBP analysis, PCB 170, 180, and 187 showed similar conditional PIPs (around 0.3). Also here the approximate posterior estimation method gives strongly biased estimates, resulting in a significant effect estimate for the association between PCB170 and DBP (Figure 10), whereas estimates obtained by the exact method are all close to zero. Similar as in the BKMR with component-wise variable selection, the overall mixture-effect for DBP appeared to be significant according to the approximate estimation method, but not according to the exact method (results not presented).

Table 4 Group and conditional PIPs from BKMR models with hierarchical variable selection.

Exposure biomarker	Group	DBP		SBP	
		GroupPIP	CondPIP	GroupPIP	CondPIP
Cd	1	0.006	1.000	0.010	1.000
Cu	2	0.002	1.000	0.002	1.000
Mn	3	0.008	1.000	0.001	1.000
Pb	4	0.005	1.000	0.009	1.000
Tl	5	0.000	NaN	0.000	NaN
OXC	6	0.090	0.280	0.027	0.422
DDE	6	0.090	0.720	0.027	0.578
TN	7	0.008	1.000	0.004	1.000
PCB118	8	0.170	0.005	0.126	0.008
PCB138	8	0.170	0.007	0.126	0.042
PCB153	8	0.170	0.040	0.126	0.076
PCB170	8	0.170	0.797	0.126	0.337
PCB180	8	0.170	0.098	0.126	0.269
PCB187	8	0.170	0.054	0.126	0.268

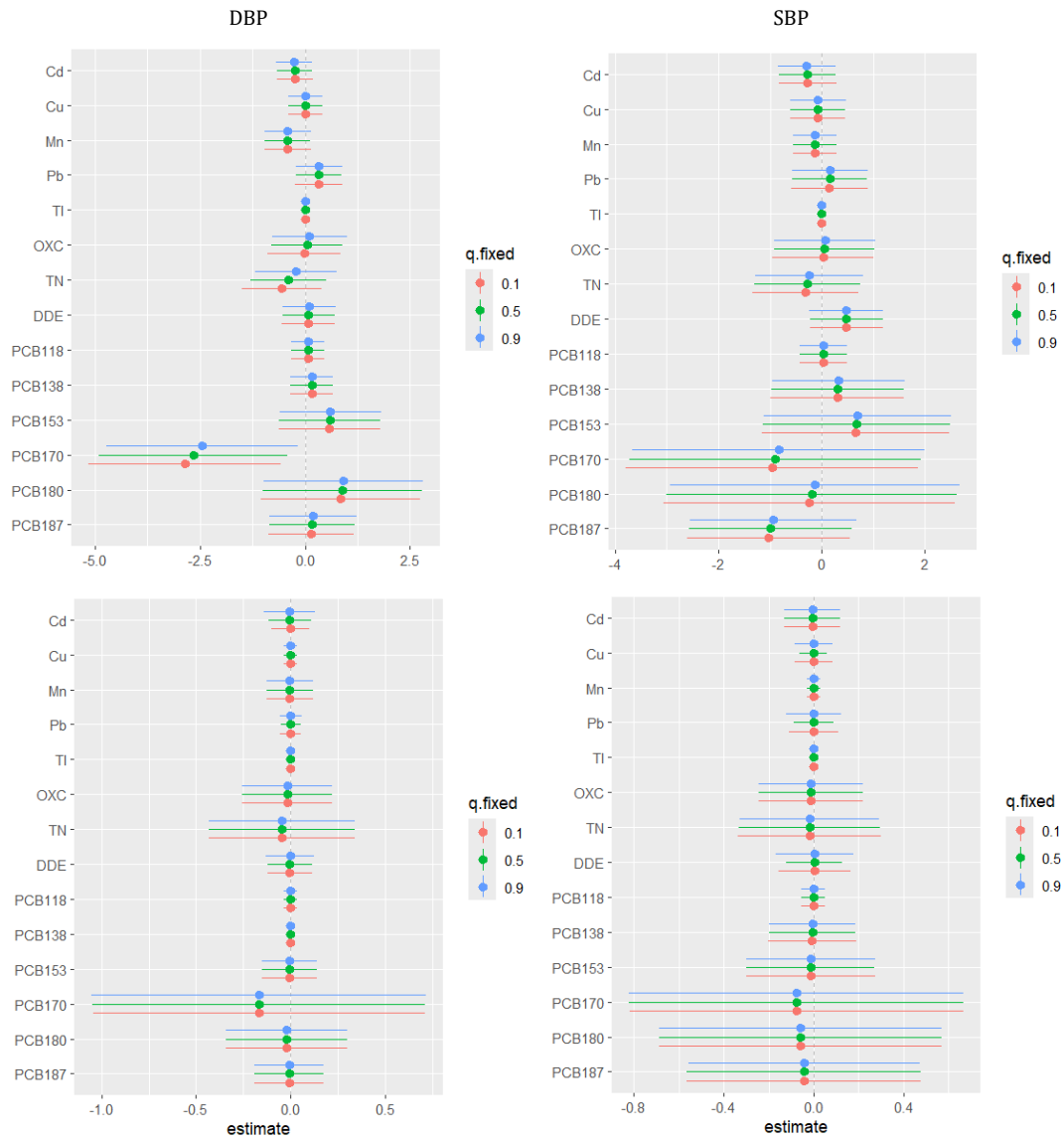


Figure 10 The estimate for an IQFc of the exposure (with 95% CrI), fixing other exposures at P10, P50, P90, estimated by BKMR with hierarchical variable selection for DBP (left panel) and SBP (right panel), using the approximate (upper panel) and exact (lower panel) estimation method.

- Conclusion: Except for the negative association between SumPCB and SBP in mpMLR, none of the multi-pollutant methods provided evidence for associations between individual exposure biomarkers and BP. QGComp, however, indicated a significant overall mixture effect for both DBP and SBP, pointing at a lower BP in association with increased mixture exposure.

1.3. Result blood pressure ESTEBAN

Data

- N= 1324 adults (from 18 years to 75 years) from the ESTEBAN French study
- Outcomes: diastolic & systolic blood pressure (DBP & SBP)
- Exposure biomarkers (36 chemicals): Metals (V, Cr, Mn, Co, Ni, Cu, Zn, As, Se, Mo, Pd, Cd, Sn, Sb, Ba, W, Pt, Hg, Tl, U, Li, Be, B, Al, Au, Cs, Ir)
- Determinants: Sex, Age, household education (ISCED)

Data treatment

- Urine samples (corrected for creatinine) and blood samples
- Only chemicals that were detected in at least 70% of the samples were considered
- Chemical measurements below the LOQ were imputed by single random imputation from a truncated lognormal distribution

Models

- Weighted quantile sum regression (WQS)
- Quantile G-computation (QGcomp)
- Elastic net (ENET)
- Bayesian model averaging (BMA)
- Random forest (RF)
- Bayesian kernel machine regression (BKMR)

Results

➤ The correlation plot did not reveal any strong correlation (Figure 11).

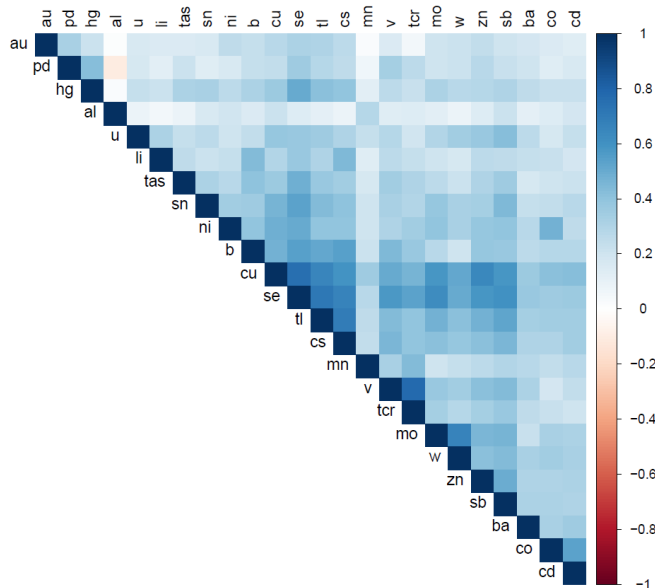


Figure 11 Spearman correlations between exposure biomarkers

6. For DBP and SBP results of **WQS** (categorizing exposure into deciles) using negative repeated holdout validation models didn't show significant mixture effect. For DBP, WQS using positive repeated holdout validation model showed a slight mixture effect (0.353 mm Hg, 95% CI: 0.106, 0.6) and highest weight was observed for thallium whereas bootstraps didn't work on SBP (Figure 12, Figure 13 and Figure 14).

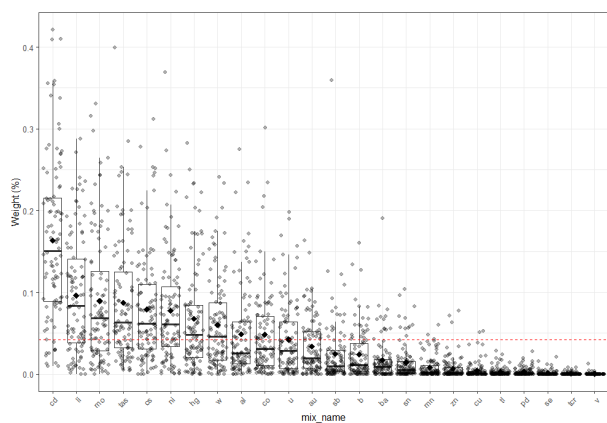


Figure 12 WQS, negative models, SBP

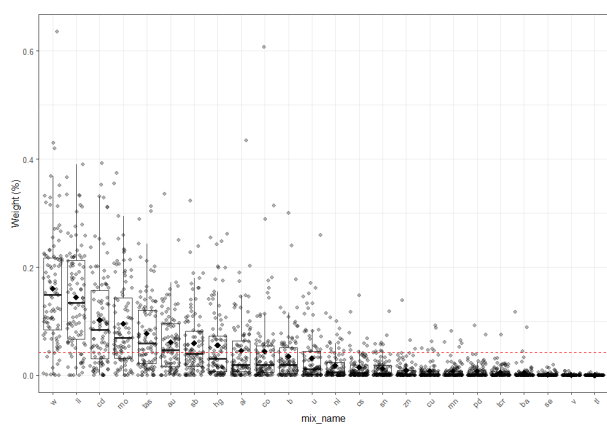


Figure 13 WQS, negative models, DBP

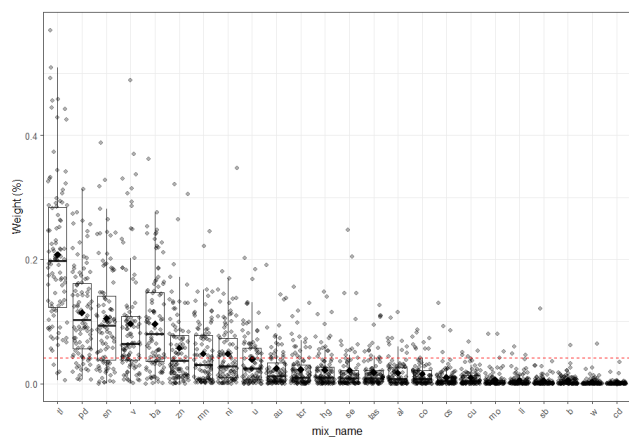


Figure 14 WQS, positive models, DBP

- The highest weight estimated with **QGcomp** was a positive weight for thallium and vanadium for DBP, and it was vanadium only for SBP. The overall mixture estimate (for a 1 decile increase in all exposure biomarkers) was not significant for both DBP (-0.34 mm HG, 95% bootstrap CI: -0.99, 0.32) and SBP (0.04 mm HG, 95% bootstrap CI: -0.37, 0.45).

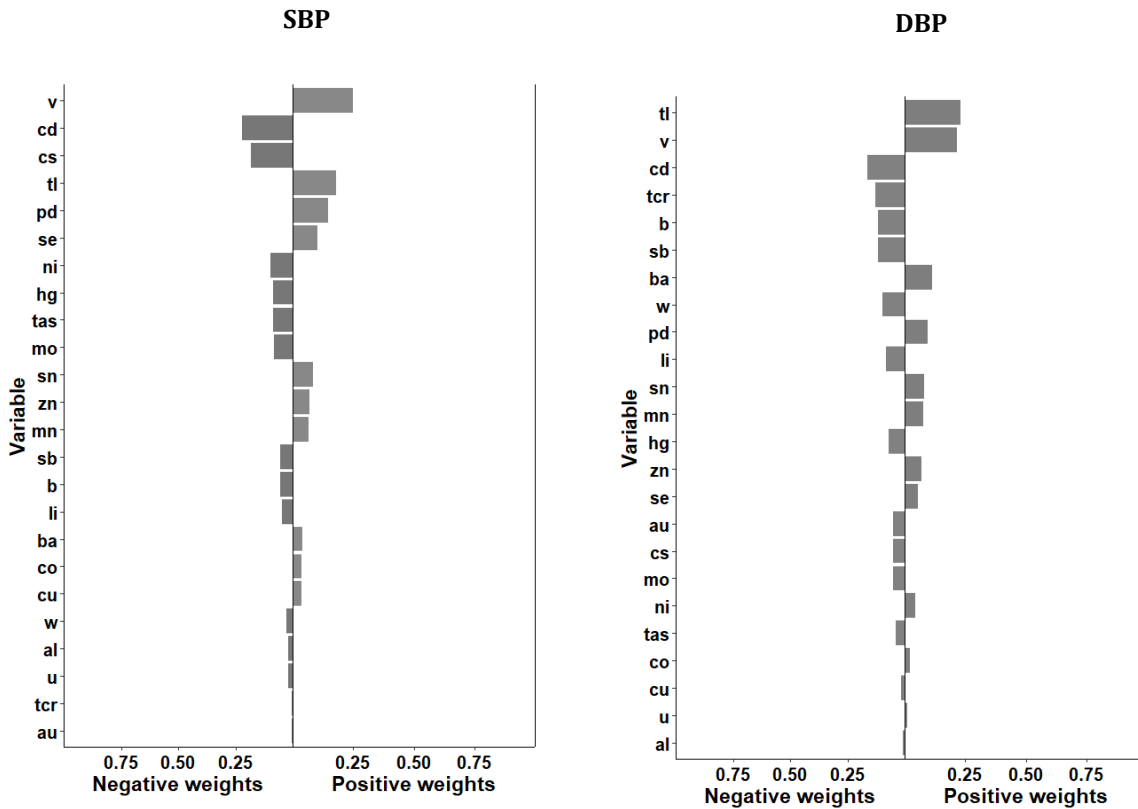


Figure 15 Weights estimated by QGcomp for the association between exposure biomarkers (categorized into deciles) and SBP (left panel) and DBP (right panel).

- Stability selection for ENET showed the highest selection frequency for vanadium, cadmium, palladium and nickel for SBP, and for thallium, vanadium and barium for DBP. However, the threshold of 0.8 was not met, so there was no strong evidence for an association between BP and any of the exposure biomarkers (Figure 16)

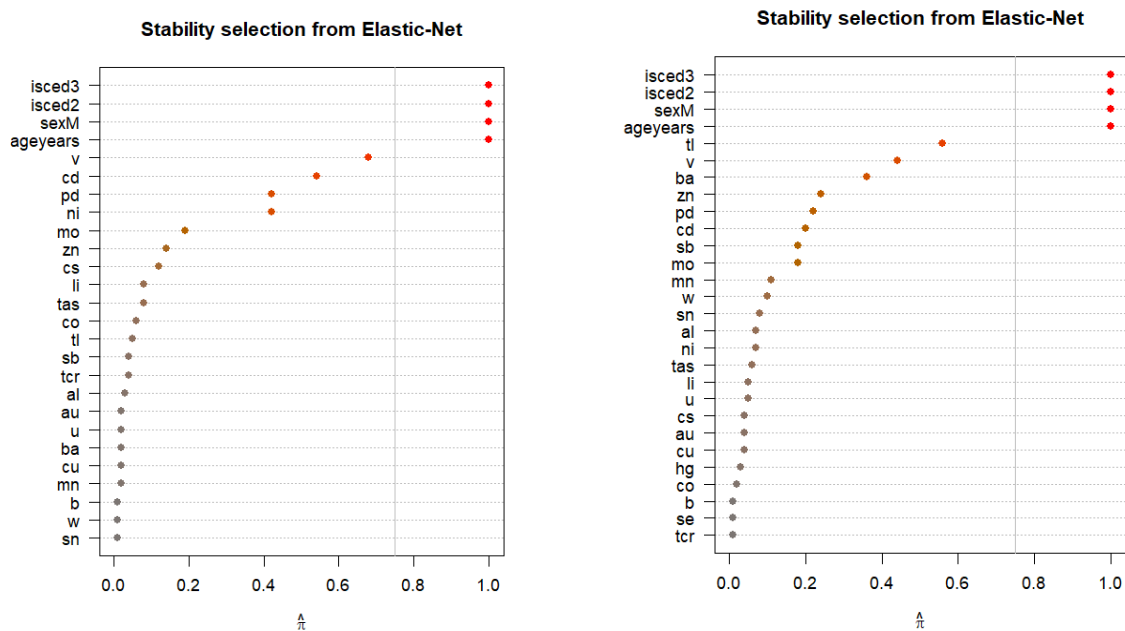


Figure 16 Stability selection for ENET models for SBP (left panel) and DBP (right panel): selection frequencies for the different exposure biomarkers, using a threshold of 0.8 for the selection frequency and targeting a per-family error rate of 0.5.

- Marginal posterior inclusion probabilities (PIPs) estimated by **BMA** were highest for cadmium for SBP and DBP but were low, indicating no evidence for an association between exposure biomarkers and BP (Table 5)

Table 5 PIP values for the 7 highest PIP of exposure biomarkers in association with DBP and SBP, estimated by BMA

DBP		SBP	
Exposure Biomarker	PIP	Exposure Biomarker	PIP
cd	0.0174	cd	0.0658
mo	0.0116	ni	0.022
hg	0.0112	v	0.0138
sb	0.0106	mo	0.0136
w	0.009	sb	0.0094
u	0.0084	hg	0.0064
cu	0.0082	zn	0.0056

- Variable importance (VIMP) of **RF** models indicated the importance of some covariates (age and sex for DBP for SBP), but did not provide strong evidence for important exposure biomarkers (Table 6). A comparison of the VIMP of pairs of variables (“paired VIMP”) with the sum of individual VIMPs (“additive VIMP”) indicated interaction effects between age and sex but did not show strong evidence for interactions between exposure biomarkers (results not shown).

Table 6 . Variable importances for the association with DBP and SBP estimated by RF

DBP			SBP		
Variable	Importance	Relative importance	Variable	Importance	Relative importance
ageyears	6.39739	1	ageyears	2.84979	1
sex	3.722804	0.581926	sex	2.128084	0.746751
v	1.527429	0.238758	v	1.311127	0.460078
ni	1.379946	0.215705	cd	1.277315	0.448214
cd	1.309226	0.20465	w	1.254433	0.440184
sn	1.293228	0.202149	hg	1.247768	0.437846
zn	1.27797	0.199764	sn	1.239858	0.43507
w	1.264975	0.197733	ba	1.232951	0.432646
mo	1.256448	0.1964	mo	1.231913	0.432282
co	1.248452	0.19515	li	1.224912	0.429825
b	1.243109	0.194315	b	1.224014	0.42951
tl	1.229899	0.19225	co	1.219277	0.427848
pd	1.225707	0.191595	u	1.214137	0.426044
u	1.225609	0.19158	al	1.203033	0.422148
hg	1.224036	0.191334	sb	1.166877	0.409461
tcr	1.222221	0.19105	zn	1.165125	0.408846

ba	1.221785	0.190982	pd	1.164718	0.408703
cu	1.221774	0.19098	au	1.160343	0.407168
mn	1.213734	0.189723	ni	1.144946	0.401765
sb	1.211757	0.189414	tl	1.140169	0.400089
tas	1.202549	0.187975	mn	1.134912	0.398244
al	1.202157	0.187914	cs	1.129323	0.396283
li	1.1867	0.185498	se	1.126691	0.395359
au	1.186142	0.18541	tas	1.124954	0.39475
cs	1.185677	0.185338	cu	1.124084	0.394444
se	1.170239	0.182925	tcr	1.122001	0.393714
iscd	1.074953	0.16803	iscd	1.062999	0.37301

- The **BKMR with component-wise variable selection** (variable selection on the individual exposures) did not converge despite the 10000 iterations (PIPs were very high).
- Conclusion: The different models applied here did not provide strong evidence for associations between metal exposure biomarkers and BP. Nevertheless, a positive association was found with WQS on DBP. Some refinements could be carried out such as raising the number of iterations for BKMR models, correcting for the age and sex interaction before applying the models or adding more cofounders related to BP (such as BMI, smoking or individual dietary salt intakes). Due to the lack of overall evidence of an association between metals and BP this associations will not be further explored. A similar work was conducted on the same study (ESTEBAN) considering metals (36 chemicals) and PCBs (53 chemicals). The study population was smaller (485 adults). Due to high correlations, PCBs were studied as a sum. The conclusions of this work were similar to the ones presented in this document.

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