CHARACTERISING CHEMICAL CO-EXPOSURES IN EU TO SUPPORT A COMBINED EXPOSURE ASSESSMENT STRATEGY

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PREFACE

This study was commissioned by the European Chemical Industry Council (CEFIC) and conducted by ARCHE Consulting and the Flemish Institute for Technological Research (VITO).

On 14 October 2020 the EU Commission published its Chemical Strategy for Sustainability1. This strategy calls to systematically integrate the issue of combined exposure (i.e. exposure to unintentional mixtures of different chemicals via our surroundings or the environment) into chemical risk assessments. This report focusses on reviewing of existing (monitoring) data and research with the aim of improving the scientific knowledge base of any future regulatory approach.

CEFIC has defined the scope and aims of the project, but ARCHE Consulting and VITO have performed all data collection, literature review and data analyses. Discussions were planned and held between the sponsor and the consultants during the course of the project. However, the content, analyses, discussion and conclusions presented in this report are the sole responsibilities of the authors. Any opinions and conclusions in this report are those of the authors and do not necessarily reflect those of CEFIC.

Project leaders were Karel Viaene and Frederik Verdonck at ARCHE Consulting, Katleen De Brouwere at VITO and Steven Van de Broeck at CEFIC. For further information, please contact Steven Van de Broeck (sva@cefic.be) or Karel Viaene (karel.viaene@arche-consulting.be).

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1. INTRODUCTION

1.1. MIXTURE TOXICITY

Chemicals have traditionally been regulated on a chemical-by-chemical basis in which the majority of regulatory frameworks set safe exposure levels for chemicals with the assumption that the chemicals are released into a pristine location (in either the body or the environment) (Notman, 2021, Bopp et al, 2019, Price et al 2012, SCHER, 2011). As a result, current **human risk assessment (HRA) and environmental risk assessment (ERA) procedures of chemicals mainly focus on exposure to individual chemicals**, considering only a single source (Kienzler et al, 2016). In reality, the natural environment consists of a mixture of naturally occurring and synthetic compounds originating from a wide variety of sources (Notman, 2021). For a long time, toxicologists, risk assessors, and regulators regarded risks from chemical mixtures as negligible, as long as exposures to all single chemicals in the cocktail were below the levels judged to be safe for each chemical alone (SCHER, 2011, Kortenkamp and Faust, 2018). However, the recognition is growing that exposure to a cocktail of chemicals may cause chemical risks not captured by single chemical evaluations (Kortenkamp and Faust, 2018, Posthuma et al, 2019a). The underlying paradigm is that **every mixture component contributes to the combination effect in proportion to its concentration/dose and individual potency/effects**, even when each component is present at levels well below its individual effect threshold. This idea has been tested in several experimental studies. The joint effect of 16 dissimilar acting biocides was investigated in a series of 24h algal growth inhibition tests (Faust et al, 2001). Low concentrations of individual mixture components, not exceeding individual NOEC values, still contributed to the observed overall toxicity. The mixture effects were best predicted using the independent action model. The alternative hypothesis of concentration additions tended to overestimate mixture toxicity (maximum difference by a factor of 3.2). Independent action predicted also better the observed mixture effects in experiments where fish were exposed to 5 different synthetical steroidal pharmaceuticals (Thrupp et all, 2018). Mixture effects on egg production was observed even though each chemical was present at levels well below its NOEC and EC01.

As a result, there is a possibility that there are instances where chemicals studied independently do not cause adverse effects, but in combination they could pose a risk to human health and the environment (Price et al, 2012).As already mentioned above, different methods have been used to predict mixture effects from mixture components. **Concentration-addition (CA)** based concepts are frequently applied by default in the regulatory context (Bopp et al, 2019), as conservative approach (e.g., Cedergreen et al. 2008). This concept works well for chemicals with common modes of action that will act jointly in this way to produce combination effects that are larger than the effects of each mixture component applied singly. The SCHER opinion (SCHER, 2011) stated that “for chemicals with different modes of action (independently acting), no robust evidence is available that exposure to a mixture of such substances is of human health or environmental concern if the individual chemicals are present at or below their zero effect levels”. Next, to concentration-addition, effects can be more than additive (**synergistic**), or less than additive (**antagonistic**) (Kortenkamp et al, 2009, Bopp et al, 2019, SCHER, 2011). Studies demonstrating synergism in mammalian test systems were evaluated by Boobis et al

(2011). From the 90 identified mixtures studies only few included quantitative estimates of low-dose synergy. Of the 6 studies that provided useful quantitative estimates of synergy, the magnitude of synergy at low doses did not exceed the levels predicted by additive models by more than a factor of 4. It still has to be seen how relevant these interactions are, how frequently they occur at relevant exposure concentrations and to what extent these effects deviate from additivity-based predictions (Bopp et al, 2019). A more recent review points out that synergisms are restricted to specific combinations of substances, and deviations of additivity are relatively small with effects rarely exceeding more than two-fold compared to what is predicted based on the additivity assumption (Martin et al. 2021).

Clear regulatory requirements for RA of mixtures of chemicals exist within regulatory frameworks for intentional mixtures such as those in formulated products (e.g. biocides and plant protection products). **However, these regulations most often do not fully cover those mixtures that occur coincidentally after the intended use of registered products** (Kienzler et al, 2016, Notman, 2021). In addition to the regulations that cover the intentional mixtures that are present in specific products, several others focus on the exposure to unintentional mixtures in an indirect way. Examples of regulations that address these types of mixtures are the Water Framework Directive (WFD), Marine Water Directive, or Air and Soil related regulations where obtaining a good status of water bodies is the goal (Kienzler et al, 2016, Posthuma et al, 2019a). In 2012, the European Commission published a communication on the combined effects of chemicals (EC, 2012), expressing concerns about the current limitations of assessing compounds individually and proposing a path forward to ensure that risks associated with chemical mixtures are properly understood and assessed. International bodies such as the World Health Organization acknowledged the need for further considering mixtures in chemical risk assessment and regulation (Meek et al, 2011) and the Organisation for Economic Co-operation and Development recently published an overview of mixture risk assessment approaches to guide regulatory authorities in setting up a risk assessment framework for unintentional mixtures (OECD 2018). On 14 October 2020, the EU Commission published its **Chemical Strategy for Sustainability**. This strategy calls to systematically **integrate the issue of combined exposure** (i.e., exposure to unintentional mixtures of different chemicals via our surroundings or the environment) into chemical risk assessments. However, this is unlikely to be an easy task as the assessment of mixtures remains challenging (Kortenkamp et al. 2009; SCHER, 2011) due to the large number of chemicals in use, combined with the limited amount of information on their toxicity and their near infinite number of possible combinations (Bopp et al, 2019). When having a closer look into RA of chemical mixtures and specific case studies, some methodological issues are recurrent. While in the US whole effluent testing approaches are quite often used (US-EPA, 2000), in the EU whole mixture effect data are available for a limited number of mixtures only, and single chemical hazard, dose–response, and Mode of Action (MoA) information needed in component-based approaches is often lacking for many chemical classes (Backhaus and Karlsson 2014; Evans et al. 2015; Bopp et al. 2016, 2018a). As a result, creating a **RA approach for unintentional chemical mixtures requires a lot of assumptions.** While this is also the case for single substance assessments, this is of particular importance for the assessment of unintentional mixtures since the uncertainties around single substance assessments are adding up when

combined risks are assessed. It should be noted that some of the uncertainties are already addressed in single substance assessment by the application of assessment factors (values between 1 and 1000). Including mixtures considerations where the Technical Guidance Document on risk assessment already indicated a "laboratory data to field impact extrapolation taking into account additive, synergistic and antagonistic effects from the presence of other substances (TGD, 2003).

Until a suitable predictive tool is available to identify mixtures of concern, it has been suggested to lower all safety limits for single chemicals by a certain factor, the so-called **Mixture Assessment Factor (MAF).** The MAF concept was initially introduced by Backhaus et al, 2010 and others to account for the possible co-existence of chemicals while assessing safe production and uses of substances under REACH. Currently, as the outcome of two workshops (the last one hosted by the Swedish Chemical Agency, KEMI and the Dutch Ministry of Infrastructure and Water Management) a majority of the invited participants (with very few industry stakeholders) supported the idea that a single generic MAF would be a pragmatic, effective and feasible way forward under REACH (KEMI.RIVM 2020). Posthuma et al (2016) suggested a MAF of 10 on the basis of the analysis of Dutch monitoring data and a preliminary assessment of direct effects on macrofauna. It was observed that for a given location 5 – 10 individual chemicals can typically be held responsible for a majority of the combined environmental toxic pressure observed. This suggests it unlikely that combined effects of chemicals in the environment are characterized by a system in which each chemical contributes an equal share to the ecotoxicity (Posthuma et al (2016). The number of chemicals assessed per sampling location varied widely from a few to 140 chemical substances. Van Broekhuizen et al (2016) stressed that these results should, however, be further evaluated for a EU representative data set of monitoring data to draw more firm conclusions and identify those chemicals that matters most.

The observation that environmental effects of **combined exposure to multiple chemicals are often dominated by a limited subset of the chemicals** is in line with the growing evidence from field studies (retrospective RA) and model predictions (prospective RA) suggesting that for a given type of effect, a large part of the combined effects from multiple chemicals in the environment is caused by a relatively small fraction of the chemicals involved (Harbers et al, 2006, Zijp et al, 2014, Backhaus and Karlsson, 2014). It should not come as a surprise that the MAF does not have to represent the number of chemicals that may potentially be present in the environment (i.e., > 100,000 compounds) to account for combined effects of chemicals in the environment. Most chemicals are present at levels that are too low to have a substantial impact on the overall combined effect (Kortenkamp and Faust, 2018). In general, mixture interactions (including antagonism, potentiation, and synergies) usually occur at medium or high dose levels (relative to the lowest effect levels). At low exposure levels, they are either unlikely to occur or are toxicologically insignificant (SCHER, 2011). As for the large majority of mixtures, the combined risk is acceptable, a relevant question to pose is rather what the best risk management approach is. The MAF should rather only account for the number of chemicals that dominate the combined effects.

It should be noted, however, that the identity of the dominant chemicals (or one dominant chemical) has also a strong spatio-temporal component (Posthuma et al, 2019a). Indeed, there is information that indicates that the scale (e.g., looking at a small pond, a river delta or a combination of deltas on a continent) is an important factor determining the total number of chemicals that may actually contribute to e.g. 90% or 99% of the effect observed (Van Broekhuizen et al, 2016).

Insight in the number of chemicals, specific chemicals or chemical groups that matter most with regard to combined effects in the environment Europe-wide is needed to **further refine and validate the MAF-concept**. Monitoring data are essential is this regard as they can provide information on magnitude, duration, frequency and/or timing of real exposure, and allow to assess the co-exposure patterns to chemicals (Qian et al., 2015). Ideally, exposure data should be aligned with ecological monitoring data and/or ecological status classifications. One of the initiatives exploring the value of using monitoring data sets is the **SOLUTIONS project** (Brack et al. 2015, 2017). This project visualized, mainly via modelling, the spatial distribution of combined effects (expressed as multi substances Potentially Affected Fraction: msPAF) of a large subset of chemicals (including biocides, pesticides, pharmaceutical chemicals, industrial chemicals), and provided an overview of those particular substances that dominate the combined predicted effects in the environment (Backhaus et al, 2019; Posthuma et al, 2019b; Posthuma et al, 2019c; Kortenkamp et al, 2019)

It should be noted that there are significant limitations using available monitoring data. For example, several authors from the selected case studies highlighted the problem of large data gaps regarding the availability of measured exposure concentrations and appropriate modelling approaches. Additionally, linking toxic effects to monitoring data can only consider chemical substances that are identified; substances that are not analyzed nor detected because they are present at concentrations below the limit of detection are not taken into account at all, although they might be biologically active (Van Broekhuizen et al, 2016, Bopp et al, 2019). The latter bias can be circumvented by performing a cumulative risk assessment with the detection limit as measured concentration. In general, statistical associations, when found, do not necessarily imply causation (Posthuma et al, 2019a). Another uncertainty is the temporal profile of co-exposure.

From the above it is clear that the eventual qualification and quantification of the MAF under REACH requires further work regarding the identification of (the number of) chemicals and uses that contribute most to the overall EU-wide combined adverse effects (Van Broekhuizen et al, 2016). To date, there is some insight into the specific chemicals, groups of chemicals, chemical classes or use conditions that dominate combined effects on the environment within Europe and it is clear that the **use of one generic "blanket" MAF for REACH registrations will not be sufficient as it will always be arbitrary, and its scientific foundation would be poor**.

1.2. IMPROVING THE SCIENTIFIC KNOWLEDGE BASE

Addressing mixture toxicity is a major challenge, both from a scientific and a regulatory perspective. Currently, scientific knowledge is being developed to help inform regulatory actions. The current study aims at **identifying critical elements for the basis for deriving the scope of applicability of a MAF** that would allow industry to comply with potential upcoming legislative obligations while ensuring, at the same time, that the outcome of the MAF approach would be scientifically robust and could be supported by the regulatory community. An overview of the different work packages of this project is given in Figure 1.

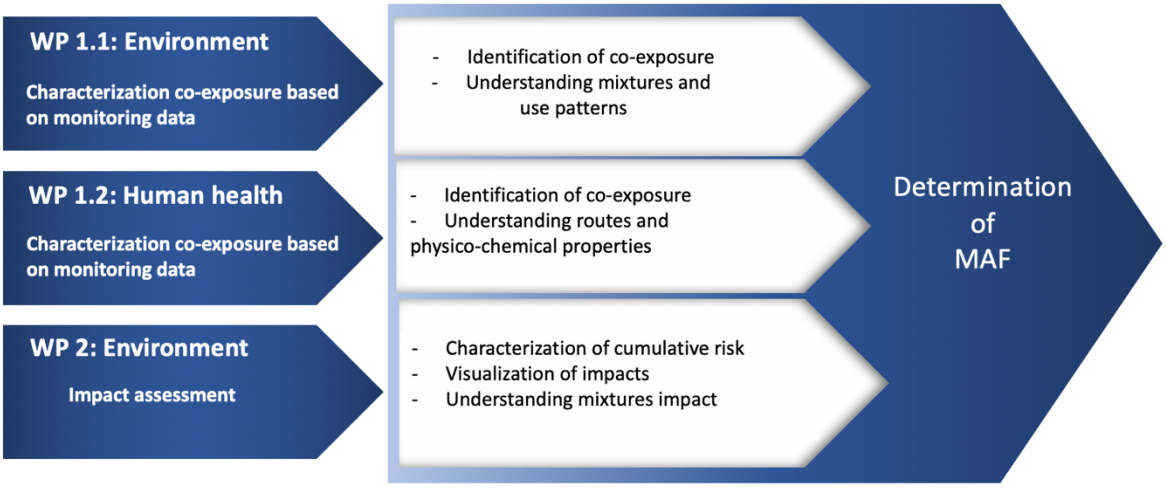

Figure 1: Overview work packages.

The different work packages **contribute to the scientific knowledge base** of mixture toxicity and bring a new perspective by starting from data available in **monitoring databases** and literature.

A first focus of this project is to understand chemical co-occurrence on a concentration basis in the European environment (WP 1.1). What are the most common mixtures in the environment, what do they look like and why do we find these mixtures? The composition of mixtures and the identification of underlying patterns are explored together with the type of chemicals (e.g., metals, PAHs, pesticides, pharmaceuticals), the regulatory background (e.g., REACH, PPP, WFD), the use patterns (e.g., household chemicals, industry chemicals, agrochemicals) and other factors (e.g., spatiotemporal). All this will help to better understand why certain mixtures are found and what we can expect for non-monitored locations. This knowledge will be key to any effective management of potential risks from combined exposures.

Exposure of humans to mixtures of chemicals is the second main concern driving the current initiatives to better regulate mixture toxicity (WP 1.2). **What are the mixtures humans are exposed to and do these pose a potential concern to human health?** Human exposure to mixtures will be investigated using human biomonitoring data and studies.

For mixture toxicity, the toxic pressure of the chemicals in the mixture is the determining factor (WP2). **What mixtures pose a potential concern and which and how many chemicals drive these potential concerns? Is potential risk the result of substances of known concern?** The mixtures at risk will be identified and further investigated for common characteristics across a range of databases. Additionally, biological monitoring data will be used to determine to what extent mixtures at risk actually lead to measurable effects on the biology. **Can we link predicted mixture toxicity to biological effects in the field?**

Central to these questions is trying to understand how chemicals co-occur, and which combinations dominate and may potentially present a potential concern. This will be the starting point for appropriate identification of uses, locations or types of chemicals which should be managed by application of a MAF. In addition, preliminary, data-driven possibilities to quantify the MAF to assess the combined toxicity of chemicals in the environment and the humans is evaluated. Starting from a wide array of monitoring databases allows a unique and realistic assessment of chemical co-exposure over a wide range of conditions.

2. ENVIRONMENT

2.1. IDENTIFICATION OF CO-EXPOSURE

The co-exposures in the EU environment have been characterized with the focus on aquatic systems using the outputs from recent EU activities such as the SOLUTIONS project database and other relevant environmental monitoring databases.

Exposure assessment based on modelling or monitoring data each have their advantages and disadvantages and it is usually recommended to be conducted in parallel (ECHA Guidance, R.16). The main advantages of exposure modelling are that it allows to characterize more (non-monitored) chemicals in mixtures (although still incomplete, e.g., 10,000 chemicals in van de Meent et al. (2020) of the ca. 150,000 chemicals on EU market), there are no issues with detection limits and modelling is not sensitive to sampling stations or times. Also, modelling can target specific sources and can therefore exclude historical contamination or natural sources. The main advantage of exposure monitoring is that – generally – monitoring data are far more accurate and realistic (compared to factor 10x-100x for modelling) because these are based on realistic spatial use pattern and realistic degradation and other fate processes. Additionally, monitoring does not require input data such as physico-chemistry characteristics or use volumes of chemicals, which need to be of sufficient quality to allow accurate modelling. In modelling, a (population-dependent) equal geographical and temporal spread of emissions is often assumed. Monitoring data inherently reflect spatial and temporal variability in use pattern. Monitoring is well suited as a higher tier approach (whereas modelling mainly works well for screening level assessments).

In the SOLUTIONS project (WP M1), it was chosen to characterize exposure to mixtures using an exposure model, rather than using monitoring data. Van Gils et al. (2020) simulated concentrations of toxic substances to chemical monitoring data for 226 substance/basin combinations which showed that the simulated concentrations were accurate on average. For 65% and 90% of substance/basin combinations, the error was within one and two orders of magnitude, respectively (van Gils et al., 2020), which is lower compared to the inter-site concentration variability spanning up to 20 orders of magnitude for the 1785 chemicals present in the analysis, and the variability of the species sensitivities, which span nine orders of magnitude when assessed by the medians of the respective Species Sensitivity Distributions (SSD). Whilst this is true and modelling is sufficiently robust for screening risk assessment, an uncertainty factor of 10 times to 100 times (ratio of modelled versus monitored concentrations) is still large in the context of regulatory discussion of proposing a targeted MAF.

In the current study, co-exposure therefore was assessed based on large and representative monitoring data sets as this is somewhat lacking in the scientific literature (e.g., Van Broekhuizen et al., 2016; Price et al., 2012). In the current project, databases were searched that includes a wide number of analytes. All databases were publicly available, either from a repository or upon request. Three types of databases were considered:

European-wide databases, river basin databases and national or regional databases. These databases were evaluated based on number of observations (both in time and space), number of chemicals measured simultaneously, use patterns of the measured chemicals (e.g., household, industrial, agricultural use chemicals) and chemical legislation of the chemicals (e.g., REACH, Plant Protection Products, BPR, pharmaceuticals). A summary of the assessment of the databases considered in the project and the final conclusion can be found in Table 1. In the SOLUTIONS project, the Danube and Rhine data sets together with four Spanish basins were used for estimating the accuracy of the modelling (van Gils et al., 2020).

Table 1: Databases for identification of co-exposure. See Technical Annex A section 1.1 for more information.

Type DB	Database	Assessment
Euro-pean	Waterbase	Good database with European wide monitoring data https://www.eea.europa.eu/data-and-maps/data/waterbase-water-quality-icm-1
	NORMAN network	Limited number of (emerging) chemicals measured simultaneously (20) https://www.norman-network.com/nds/
	IPChem	Collection of individual datasets, not a homogenous database https://ipchem.jrc.ec.europa.eu/index.html#discovery
River basin	Danube	Limited number of sites (40) compared to number of chemicals (395) limit statistical analysis https://www.icpdr.org/wq-db/
	Rhine	Good database with several sites monitored over many years https://www.iksr.org/en/topics/water-quality/water-quality-data
National or Regional	EauFrance	Good database with many sites and a wide range of chemicals https://geo.data.gouv.fr/fr/datasets/5a3bfb08ce37de663133c8c19034231b4fc6d76c http://www.naiades.eaufrance.fr/france-entiere#/
	Flanders (BE)	Good database, focused on priority pollutants http://geoloket.vmm.be/Geoviews/
	The Netherlands	Good database, focused on PPP https://www.waterkwaliteitsportaal.nl/wkp.webapplication
	Sweden water	Only water chemistry parameters, cations/anions http://www.slu.se/miljodata-MVM https://www.slu.se/en/departments/aquatic-sciences-assessment/
	Sweden pesticide	Limited number of locations (6), only pesticides
	UK	Limited number of chemicals (6 PAHs, 6 agrochemicals) https://data.gov.uk/dataset/bda4e065-41e5-4b78-b405-41c1d3606225/historic-uk-water-quality-sampling-harmonised-monitoring-scheme-summary-data

The EU Waterbase, Rhine and EauFrance databases were selected for further data analysis as the most relevant and useful databases for assessing co-exposure. More detailed information on the databases and the assessment (e.g., size, measured parameters, criteria for inclusion in the analysis) can be found in Technical Annexes A (section 1.1) and B.

When discussing environmental monitoring databases, it is important to avoid confusions related to the terminology used. In this report, if no chemical analysis was performed to determine the concentration, the chemical is defined as a **non-measurement**. **Non-detects** refer to the situations where a chemical analysis was performed, but chemical was not detected (below the limit of detection).

Several observations can be made considering the measurements in all the databases included in the analysis:

- The **majority of the chemicals are not detected**. Depending on the database, 50-80% of the chemicals are either not present or present at very low concentration (below the detection limit) in the observed mixtures (see Figure 2: Percentage of detected and non-detected chemical in several chemical monitoring databases (all samples)).
- A limited set of chemicals is nearly always included in the monitoring strategies (mainly priority pollutants, consisting of metals, polyaromatic hydrocarbons and pesticides) but the monitoring of the other chemicals is more heterogenous and is dependent on the scope of each monitoring database. **How to deal with non-measurements** when defining mixtures and how to balance the number of measurements with the number of chemicals measured is an important challenge for any analysis of the databases. The main focus of this project will be to analyze patterns and trends of chemicals that are detected (detects).
- Because of the large number of non-detects, **the approach to deal with limit of quantification and limit of detection** is another important consideration for data analysis.

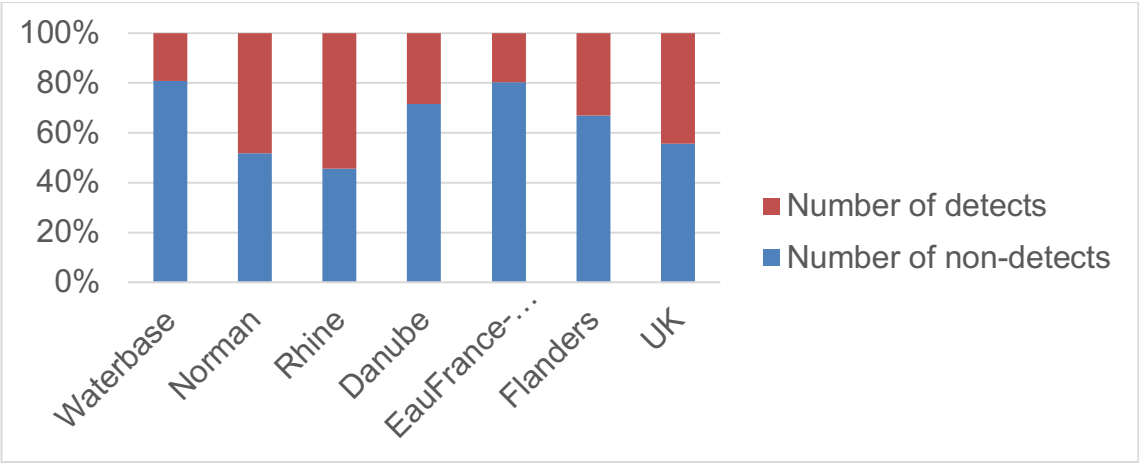

Figure 2: Percentage of detected and non-detected chemical in several chemical monitoring databases (all samples).

For those chemicals that are measured and detected, the next question is to assess which chemicals typically co-occur in observed mixtures. Summary graphs and tables have been developed that show which chemicals co-occur on a concentration basis (see Annex B, section observed mixtures for each database). For this, two techniques have been employed:

- **Principal component analysis** is a multivariate technique that allows to identify chemicals that typically co-occur on a concentration basis. So-called biplots are two-dimensional projections of a multi-dimensional space of chemical concentrations.
- **Correlation analysis** allows to assess correlation/co-occurrence between pairs of chemicals. This technique is therefore less powerful than multivariate principal component analysis but has the advantage to allow easier interpretation of the relation between pairs of chemicals and to easily identify possible causes for co-occurrence (e.g., shared use, same source, parent compound and degradation product).

Both techniques are robust against any assumption made for the non-detects because the techniques are focused on detecting correlations and relationships between chemical concentrations. The relative relation within and between the chemical observations are more determining than their absolute values. Hence, assumptions on the non-detects, e.g. using the detection limit, half of the detection limit or permutation methods to estimate values below the detection limit, do not have a major impact on the outcome of the analyses (rationale, see Annex A, section 1.2.2).

The summary graphs and tables for various data sets and the more detailed description of the methodology and results can be found per database in Annex A-C. The large number of chemicals were grouped in main use categories pharmaceuticals, industrial chemicals, household chemicals and agrochemicals to allow better understanding of the general trends (see

Table 2) and to make the link with use patterns in the next step. Note again that not all chemical groups have been monitored in all databases. Because of the size of the datasets, it is practically unfeasible to include all data in this report or technical annexes, but both raw data and the processed datasets are available upon request.

Table 2: Overview of principal components of the PCA analyses.

Database	Number of chemicals considered*	Main chemical groups in the principal components (PC)
EauFrance – whole of France	436	MIXTURE1A: Agrochemicals and MIXTURE1B industry/household chemicals MIXTURE2: (legacy) Agrochemicals MIXTURE3: Agrochemicals
EauFrance – Adour Garonne	82	MIXTURE1A: Pharmaceuticals do typically not co-occur with MIX 1BMIXTURE1B industry MIXTURE2: Mainly industry/households MIXTURE3: Agrochemicals/industry
Waterbase	72	MIXTURE1: Agrochemicals/industrial/metals MIXTURE2: Agrochemicals/industrial MIXTURE3: Agrochemicals
Rhine	43	MIXTURE1: Industrial chemicals/agrochemicals/metals/PAH MIXTURE2: Agrochemicals MIXTURE3: Agrochemicals/PAH
The Netherlands	86	MIXTURE1 & MIXTURE2: Agrochemicals
Flanders	36	MIXTURE1: Polyaromatic hydrocarbons MIXTURE2: Mainly agrochemicals MIXTURE3: Industrial/others
Norman	19	MIXTURE1: Pharmaceuticals/herbicides MIXTURE2A: Pharmaceuticals and herbicides do typically not co-occur with MIXTURE 2B: industrial chemicals MIXTURE3: Pharmaceuticals/herbicides/Plastic additives

* After selection to build a complete dataset (i.e., without non-measurements)

Considering all databases, typical mixtures consist of a combination of agrochemicals, industrial, household and pharmaceuticals. Especially the first principal component of the PCA analysis is typically a mix of a wide number of chemical groups. This indicates that **most of the mixtures in the databases are made up of a wide range of chemicals** and not determined by specific chemical groups (only exception are agrochemicals mixtures likely because of upstream agricultural areas). The EauFrance dataset seems to be the only dataset to contain a more representative distribution of REACH chemicals, agrochemicals, pharmaceuticals and others. However, the focus of most other databases is typically on priority pollutants or prioritized chemicals based on single chemical potential hazard or risk to the environment, not particularly REACH chemicals. This hinders drawing strong general conclusions across databases on mixtures consisting of only REACH chemicals. On the other hand, this does reflect the reality where potential mixture risk is not limited to mixtures of REACH chemicals/uses alone. As confirmed by the monitoring data, the chemicals regulated under REACH are typically part of broader mixture with chemicals from other EU chemical legislations and/or historical sources. This leads to the conclusion that implementation of a **MAF** is to be considered in a **broader perspective than the REACH Regulation alone**.

Within databases, more specific mixtures can be found, typically related to the properties of the database or geographic coverage and spatial variations in land use and use patterns of chemicals. For example, the

database for the Netherlands is mostly populated with agrochemicals, therefore the resulting mixtures are mostly formed by sets of agrochemicals. On the other hand, the Adour Garonne dataset contains many pharmaceuticals, resulting in the distinction between mixtures of pharmaceuticals and industry chemicals. Mixtures with a specific composition can be **more easily identified from regional databases** such as Adour-Garonne, Flanders or the Netherlands. This is not surprising since databases covering a wide range of locations will contain a larger collection of mixture compositions and will thus not (likely) show one or a few chemicals being dominant in the mixtures. Regional, more focused databases will show a smaller range of conditions (e.g., land use, population density) and a clearer distinction between mixture compositions. Additionally, datasets with a small spatiotemporal scale can be expected to better capture chemicals that only occur episodically in the environment (e.g., after a rain event). The smaller **spatial and/or temporal scale is thus an important determinant for the mixture composition, which ideally should be considered in the regulatory framework.**

These findings are in line with studies on the composition of complex mixtures in the field. Pesticides and pharmaceuticals seem to be the most measured and detected chemicals (Busch et al. 2016). Beckers et al. 2018 studied 145 organic chemicals in wastewater treatment plant discharges and downstream sites. They found exposure concentrations of pharmaceuticals and industrial chemicals were nearly constant in mixture, while pesticides and more seasonally-used pharmaceuticals were more variable in their contribution to the mixture, dependent on the season and rain patterns.

The monitoring databases are only covering a small subset of the chemicals that could be present in the mixture. It is impossible to accurately measure all potential chemicals in the mixtures, but the lack of data on unmeasured chemicals will not alter the main conclusions on co-exposure: **co-exposure will be defined by a wide range of chemicals not all covered by the REACH regulation and that the regional conditions to a large extent determine the composition of the mixture.**

Many factors can determine the mixtures found in the databases, and some are an artifact of the monitoring strategies e.g., because of the focus on monitoring priority pollutants or hot spots. However, despite the limitations of monitoring databases, we do find indications that co-exposure is dependent on the location and time of monitoring, which are in turn an **indication of the underlying use and emission pattern of the chemicals and differentiated management approaches**. This will be explored in more detail in the next section.

2.2. UNDERSTANDING MIXTURES AND USE PATTERN

Next, the common use patterns have been assessed for the identified typical mixtures. Several hypotheses can be put forward: typical mixtures or **co-occurrence of chemicals can be explained** by (see Figure 3):

- The **use pattern** (sources of household, industry, agricultural use), e.g., presence of certain pharmaceuticals is likely long-term use, and would be found downstream of WWTP, e.g., some pesticides would be found near agricultural operations. The use pattern is also related to the chemical legislation (REACH, PPP, pharmaceuticals, etc…)
- **Spatial factors**, e.g., upstream river catchments would result in less complex mixtures, downstream river catchments result in more complex mixtures both in terms of number of chemicals as well as different type of chemicals/sources/use pattern related to human activity in the given watershed.
- **Temporal factors**, e.g., agrochemicals may only be present seasonally as applied to a given crop.
- Whilst all hypotheses will likely have impact on the co-occurrence of chemicals, analysis of the various data sets demonstrated that typical mixtures are mostly explained by their use pattern and spatial factors:

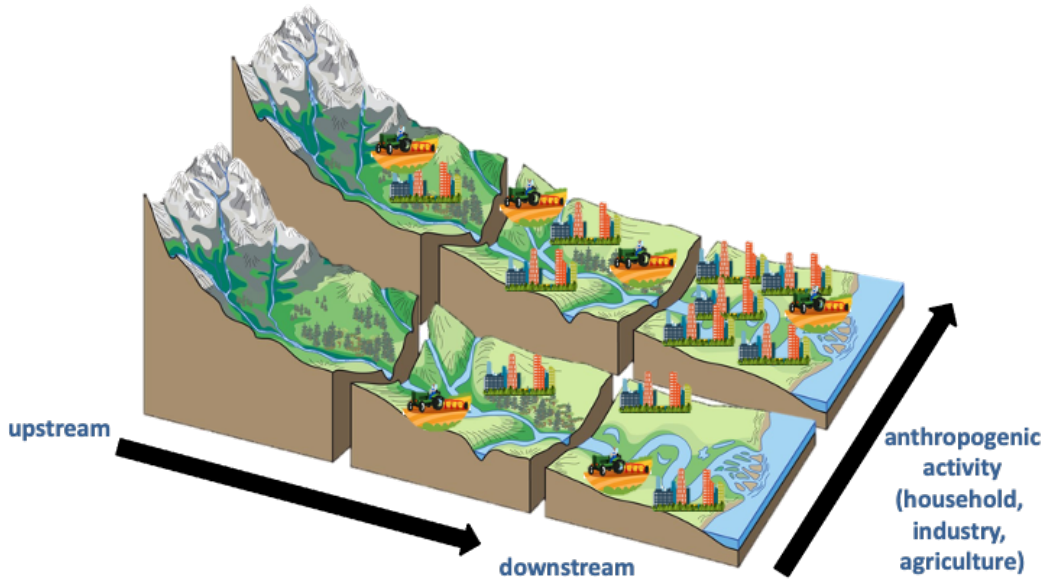

Figure 3: Chemical co-occurrence depends on chemical use pattern, spatial and temporal factors.

In the Rhine dataset (Technical Annex B 2.2), the most upstream monitoring locations (Weil Am Rhein and Lauterbourg-Karlsruhe) are largely characterized by agrochemical mixtures (likely caused by agricultural activities in Switzerland, South-West Germany and North-East France) (see Figure 4: left: the Rhine basin; right: PCA biplot indicating principal components with mixture concentration of industrial, consumer and

agrochemicals increasing from right to left and mixture concentrations of agrochemicals increasing from middle to top or bottom (color codes refer to monitoring locations), black arrow refers to the upstream downstream sequence). As one moves more downstream the Rhine river passing through the Ruhr area in Germany, the mixtures become more complex (higher number of chemicals), some agrochemical exposure level is decreased (diluted and/or other multiple fate and dissipation pathways) and additional concentration increases are observed for industrial and household chemicals in the mixtures (at monitoring locations Lauterbourg, Koblenz and Bimmen). These mixtures are further diluted downstream and additional (different) agrochemicals are observed in the observed mixtures in the monitoring locations Maassluis, Kampen and Lobith (likely due to agricultural activities in The Netherlands and West-Germany). Since the Rhine dataset is focused on the main river (and not the smaller tributaries), the most downstream sites can be considered as “hotspots” regarding chemical co-exposure. The Rhine dataset also allowed to assess possible temporal factors, but it turns out that changes throughout the year (e.g., due to seasonal use effects) do not significantly change the chemical composition of the mixtures although they do impact the absolute concentrations.

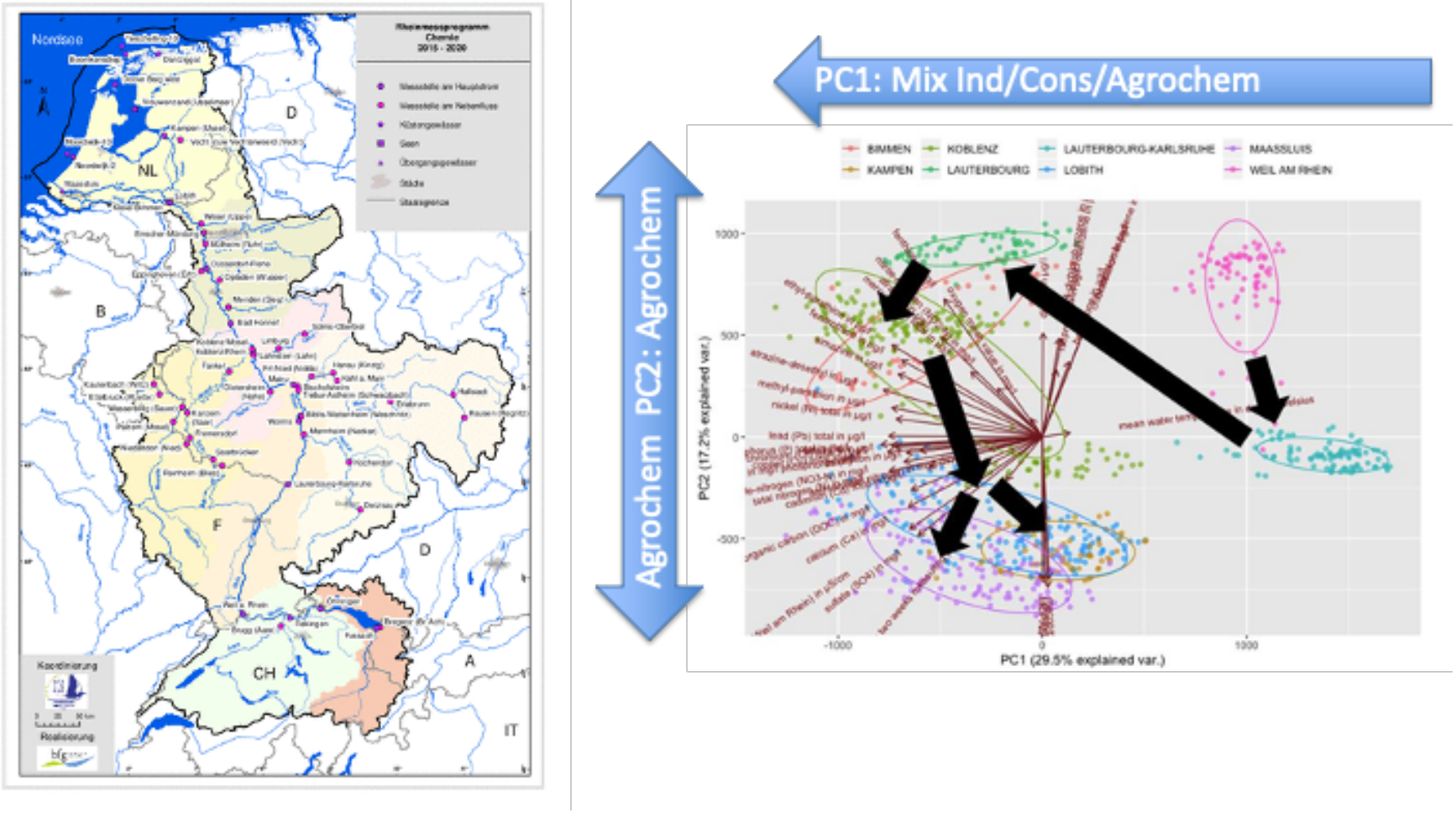

Figure 4: left: the Rhine basin; right: PCA biplot indicating principal components with mixture concentration of industrial, consumer and agrochemicals increasing from right to left and mixture concentrations of agrochemicals increasing from middle to top or bottom (color codes refer to monitoring locations), black arrow refers to the upstream downstream sequence

In the Adour-Garonne (France) dataset (Technical Annex B 2.3), the pattern is more complex to interpret due to its dense river system. The basin Adour-Garonne is divided into five sub-basins: Charente, Dordogne, Lot, Tarn-Averyron, Garonne, Adour and Côtiers aquitains et Charentais as illustrated in Figure 5.

Figure 5: Geographical illustration of Adour-Garonne basin.

The main pattern observed from this dataset is that in more densely populated regions, the mixture becomes more complex (see Figure 6). An example can be taken for mixture 1A – which consist mainly of pharmaceutical substances (See Technical Annex B 2.3.2.2. for more details). The concentration of mixture 1A in sub-basins Garonne and Tarn-Averyron is observed to be noticeably higher than other zones. Whereas the lowest pressure (i.e., concentrations) of mixture 1A is observed in Lot and Adour regions – which are lower in population compared to the other zones. It should be noted that Lot and Adour regions contain dense tributary networks which are originally from Massif Central and Pyrenée, not from the accumulation of water networks from other sub-basins. On the other hand, Garonne and Tarn-Averyron are higher populated with the presence of big cities (eg. Toulouse) and intense rural areas. Garonne is a big river which has large catchment system along the whole zone, which starts from Spain. Subsequently, spatial factor and use pattern (which mainly derived by the population) are considered as main drivers of the separation observed in this dataset.

Figure 6: left: PCA biplot indicating first principal component with mixtures that are either dominated by industrial chemicals (left) or either by pharmaceuticals (right) and second principal component with mixtures with higher (top) or lower (bottom) concentrations of industrial and household chemicals, right: map of Adour-Garonne (France) indicating sites following clusters in the PCA biplot.

In the EU Waterbase dataset (Technical Annex B 2.1), sites from France contained the highest number of measured chemicals. After data selection for the most complete mixtures (maximum number of measured chemicals), only the sites from France were retained to investigate co-exposure patterns. The mixtures found with the PCA analysis were clearly correlated with the geographical locations. Both the regions and the river basins containing monitoring sites were distinct in the PCA biplots, indicating the importance of the spatial dimension. Sites in the river basins of the Scheldt, Meuse and Rhin as well as sites near the North Sea cluster together. These areas are densely populated, historically heavily industrialized and characterized by intensive agriculture, mainly for food crops. Mixtures found for these locations have similar profiles, defined by a mixture of agrochemicals, metals and industry metals or by distinct mixtures of agrochemicals. Sites located in the area around the Seine and in Normandy overlap to a large extent with this cluster, but also contain distinct sites that do not overlap and that thus had different chemical co-exposures. This was attributed to differences in agricultural practices i.e., the sites in Normandy are more focused on livestock production and less heavily industrialized than sites close to the Rhine and Meuse. Similarly, the Rhône only shows a partial overlap, associated with industrial activities around major city centers (e.g., Lyon) and the mixed agriculture in the rest

of the region (mix of field crops, livestock and vineyards). Sites in the Loire river basin and in Brittany are very diverse in the type and intensity of the land use, resulting in a wide range of chemical compositions.

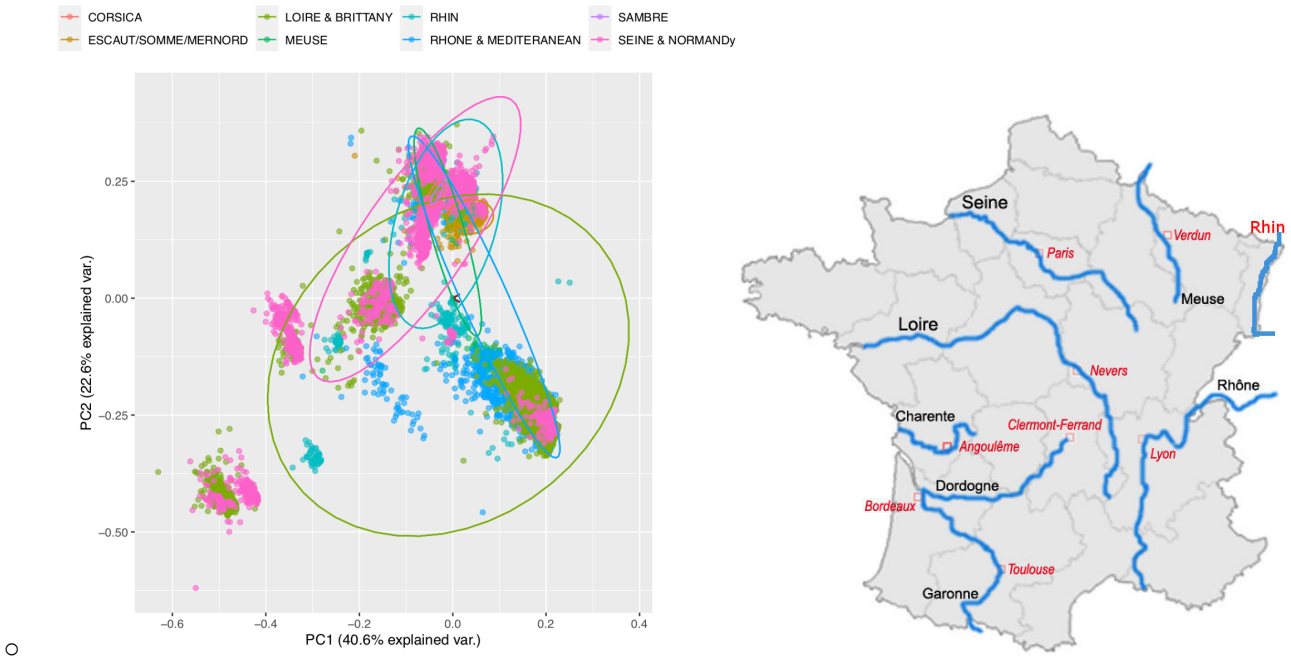

Figure 7: PCA biplot with color code clustering for the river basins in the final Waterbase dataset (left). Lines indicate in which area 95% of the sites fall. Map or France with most important rivers (right).

This is in correspondence with observations that toxic pressure is mainly driven by population centers and economic activities and dilution capacity (van Gils et al., 2019). Van Gils et al. (2020) (and more broadly the SOLUTIONS project) looked at mixture toxic pressure by considering a Europe-wide integrated exposure and effect modelling, explicitly considering a spatially and temporally resolved model for emissions and fate-and-transport of chemicals. Similarly, Beckers et al. 2018 found that exposure to pharmaceutical and industrial chemicals were more constant in mixtures downstream from a WWTP, reflecting the urban emissions. Pesticides were more seasonally driven and depended largely on the run-off by rainfall. Munz et al. 2017 is in line with these findings, showing higher water concentrations of pharmaceuticals and household chemicals downstream of WWTPs, although the risk was mainly determined by pesticides. Mixtures in the field have also be linked to nearby sources and land use for estuaries (Mijangos et al. 2018) or rivers (Beckers).

It can be concluded that the **complexity of the mixtures is largely driven by the spatial location and the intensity of anthropogenic activity** (household, industry, agriculture). The more downstream a river catchment, the more complex mixtures can be expected both in terms of number of chemicals as well as types of chemicals. Reversely, more simple mixtures can be expected more upstream river catchments. The **REACH** Regulation largely relies on so-called **generic exposure scenarios** leaving out spatial character of environmental exposure. This **complicates the refinement options in higher tier approaches** following

application of a generic MAF factor under the REACH Regulation. It is noted that other European legislations, such as the Water Framework Directive, have a more spatial oriented framework than single chemical driven legislations in REACH.

2.3. CHARACTERISATION OF PREDICTED CUMULATIVE RISK

The focus for the characterization of predicted cumulative risk was on those datasets (i.e., the Rhine dataset and the Adour-Garonne dataset) that support the identification of mixtures of concern for predicted cumulative risk for a broad range of substances and/or observed ecological risk (effects identified).

Cumulative risk of a mixture can be relatively straightforward predicted with the **concentration addition approach (CA).** This approach assumes that substances act via a common mode of action. In addition, it is assumed that all substances contribute to the mixture pressure, even at very low concentrations, proportionally to its Toxic Unit (i.e., conc/effect threshold level). The concentration addition model is generally considered to be a conservative approach (e.g., Cedergreen et al. 2008). Although the TU method has originally been developed for summing TU at the species level (i.e., using effect levels), in the context of environmental regulation, the concept is often combined to sum Toxic Units based on environmental threshold levels such as PNECs or HC5s (i.e., sum of ratios of concentration/PNEC or concentration/HC5), and is under this form preferred as a first conservative tier when assessing cumulative risks (OECD 2018).

In the present study, the **Hazard Quotient (HQ)/Hazard Index (HI)** approach has been used to estimate cumulative risks (Price et al. 2012, OECD 2018). The Hazard Quotient (HQ) is the ratio of an environmental concentration relative to the environmental threshold, such as the HC5 or PNEC, for an individual substance (Eq. 1). The environmental threshold level selected for the present study is the HC5. PNECs were deemed less appropriate to understand mixture impacts because (1) PNECs are not predictive of effects as they imply a regulatory based uncertainty assessment in addition to the effect assessment and (2) The use of PEC/PNEC ratios is less scientifically correct and considered slightly more conservative, however suitable as Tier 1 approach (SCHER, SCCS, SCENIHR (2012). The Hazard Index (HI) is the summation of the HQ of all substances in the mixture (Eq. 2).

$$HQ_i = \frac{c_i}{HC5_i} \qquad \text{(Eq. 1).}$$

$$HI = \sum HQ_i \qquad \text{(Eq. 2)}$$

To differentiate between risks driven by individual substances and mixtures, the concept of Maximum Cumulative Ratio (MCR) has been introduced by Price & Han (2011). The MCR is the ratio of the total toxicity to the largest toxicity from a single chemical stressor. The MCR can range from 1 to *n* (with *n* the number of chemicals in the exposure). Values close to 1 indicate that one chemical dominates the mixture toxic pressure. An MCR equal to *n* indicates that the receptor is exposed to equitoxic doses of all chemicals.

The **chronic HC5 values** were calculated based on SSD-parameters (i.e., median chronic NOEC/EC10 and log-normal SSD-slope) published in the database of Posthuma et al. (2019). These authors published parameters of both acute and chronic SSDs for over 12 000 chemical substances. Despite some concerns with Posthuma's approach, it allows high-throughput assessments of mixture risks (Posthuma et al. 2019, 2020). Concerns are, for instance, related to the lack of transparency in the SSDs of Posthuma et al. (2019), because there is no access to the toxicity data underlying the SSDs. In addition, due to the extent of the dataset, the toxicity data underlying the SSD-parameters may not have undergone the same data quality scrutiny assessment as in the substance-specific effect assessments for regulatory compliance assessment, such as in the REACH framework. The SSD-approach taken by Posthuma et al. (2019) deviates from the approach put forward in the Technical Guidance Document on Risk Assessment, because toxicity data were averaged across taxa (mainly taxonomical Classes and Orders) rather than species, neither was the most sensitive endpoint selected.

Because true synergisms are rare and mostly not occurring at the lower effect levels relevant for environmental risk assessment (Cedergreen, 2014), the Hazard Index/Hazard Quotient approach has been proposed as a conservative first tier in environmental risk (Backhaus & Faust 2012, OECD 2018). Posthuma et al. (2019, 2020) linked the Hazard Index approach with a log-normal SSD-function to express mixture pressure as multi substance Potentially Affected Fraction (msPAF). Several alternative methods exist which have been proposed to be incorporated in higher tiers of a tiered risk assessment framework. One of those alternatives is summing Toxic Units at the species level or trophic level, which is conceptually more in line with the actual concentration addition mixture reference model, because this model applies to single species and not communities (SSDs). OECD (2018) for instance has proposed to apply a concentration addition-based mixture assessment approach for the three trophic levels (fish, invertebrate and algae). Such a refinement of the mixture assessment was not possible in the current assessment, because the data needed for such mixture pressure calculations was not available from the original effects data source (Posthuma et al. 2019). As it can be expected that the underlying assumption of the concentration addition mixture reference model – all substances in the mixtures have a common mode of action – is often violated for complex environmental mixtures, the competing concept of independent action (IA), may be used in higher tier assessments, when there is an indication of multiple modes of action (e.g., Backhaus & Faust 2012). When applied on SSDs of the individual mixture constituents, the IA calculates the mixture pressure expressed as msPAF based on the product of the fraction of species not affected by each of the individual mixture constituents (Traas et al. 2002). A hybrid method ($msPAF_{MoA}$) combines the hazard index approach for substances with the same mode of action with the independent action model to calculate an msPAF for complex mixtures with both shared and different modes of actions (Traas et al. 2002; De Zwart and Posthuma 2005, Parkerton et al. 2018), such as in the environmental samples under study.

The current approach with a first level assessment using the Hazard Index approach based on summing of TU of HC5s builds in **several levels of conservatism** relative to:

1) The **use of HC5s from Posthuma et al. (2019)** instead of PNECs: due to the incorporation of an assessment factor, PNECs tend to be lower than the HC5s used in the present study. Hence, when using PNECs, risks will be 'flagged' at lower concentrations. However, this depends on the (group of) chemicals: **for some of the driving chemicals**, the HC5 derived from the dataset of Posthuma et al. (2019) appeared to be **very conservative relative to other sources**. For instance, for the polycyclic aromatic hydrocarbon benzo(ghi)perylene, the HC5 derived from Posthuma et al. (2019) was observed to be 100-fold lower than the PNEC value derived based on the target lipid model (Redman et al. 2017). Refining the Hazard Index/Hazard Quotient approach using target lipid model based PNECs for PAH, while using the HC5s derived from Posthuma et al. (2019) for all other substances, resulted in substantially lower risk predictions. While 54% of the mixture exposures were predicted at risk in the initial analysis for the Rhine dataset, after the refinement of the effect levels for PAHs, only 16% of the mixture exposures were predicted to be at risk. Further refinements on the key contributors (e.g., by considering toxic mode of action) is likely to further reduce the number of combined exposures of potential concern. This example clearly shows that the dataset of Posthuma et al. (2019) can be used as a first tier assessment, but that further refinements in the methodology can shed a different light on actual (absolute) risks.
2) **Bioavailability of inorganics and organics not included in approach**: the bioavailability and toxicity of both inorganics and organics can be dependent on the characteristics of the receiving water. For instance, the toxicity of ionizable organic compounds (IOCs) is highly dependent on pH, mainly because the uptake and bioaccumulation of IOCs is pH dependent (Escher et al. 2020). Another example are metals, for which toxicity is highly dependent on physico-chemistry characteristics such as Dissolved Organic Carbon, pH, and hardness (Mebane et al. 2020). Using generic environmental threshold levels for cumulative risk assessments typically leads to a certain degree of conservatism relative to site-specific environmental threshold levels. These site-specific environmental threshold levels may for instance be calculated with ion-trapping and toxicokinetic models (IOCs) or biotic ligand models (metals). However, site-specific bioavailability corrections are often 'data-hungry' and complex requiring expert-level knowledge, although simplified models are available for certain compounds.
3) Additional conservatism is incorporated in the assessment by applying the **concentration addition method on an environmental threshold level** such as the HC5. It has been shown that this method incorporates a substantial margin of safety relative to complex methods such as applying the concentration addition method at the species or trophic level or relative to the independent action model (Backhaus & Faust 2012, Gregorio et al. 2013, Van Regenmortel et al. 2017, Nys et al. 2017). Figure 8 demonstrates that additivity for all chemicals is quite conservative assumption as many chemicals have different mode of action in between independent action could be assumed.

   The use of environmental threshold levels, such as PNEC and HC5, in mixture risk assessment, is associated with inconsistencies as these values may be derived from different species, endpoints and, in the case of PNECs, with different assessment factors. Therefore, the OECD has recommended to apply TU refinements at the species level (Tier 1) or refinement based on MoA (Tier 2) in higher tiers

of the cumulative risk assessment process. It is demonstrated in Annex C that several risk driving substances have different toxic mode of action and that the number of mixtures of potential concern reduced from 50 to 12 for the Rhine data set (Annex C Section 3.2.5) and from 19 to 3 in the Adour Garonne data set (Annex C Section 3.3.3). In conclusion, taking into account toxic mode of action could further reduce the number of mixtures of potential concern by 75-85%.

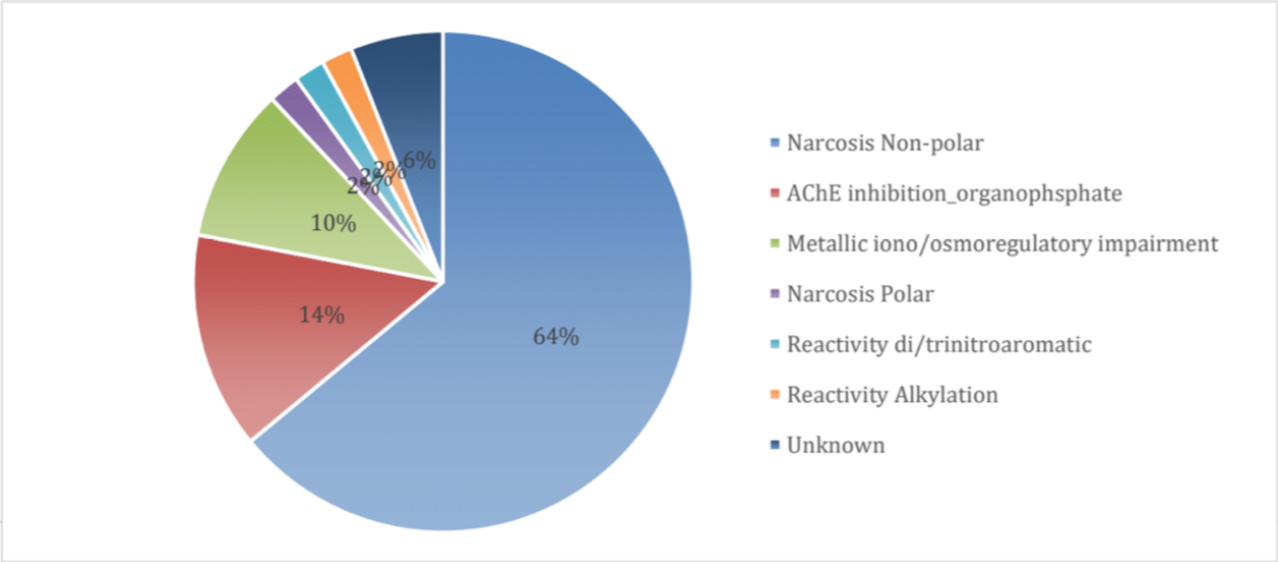

Figure 8: The different mode of actions in the mixtures observed in the Rhine basin demonstrates that a toxic unit approach is a conservative lower tier assessment as in reality, additivity is only to be assumed for substances in mixtures with the same mode of action. Percentage indicates the percentage of substances with a specific mode of action.

It is difficult to analyze the cumulative risks for a dataset if a monitored chemical is **not detected**, especially when the majority of the samples are not detected. Such a result does not prove that the compound is not present or absent, it only shows that the concentration is somewhere between zero and the LOD (Limit of Detection). Assuming a zero concentration for all non-detects will therefore underestimate the total risk, if no additional knowledge about e.g., emission or use pattern is available (Gustavson et al., 2017). On the other hand, assuming that all non-detected compounds are present at a concentration just below their LOD – the worst-case scenario that is still compatible with the recorded values – is also unrealistic. Such an approach immediately leads to the logical inconsistency that the estimated risk becomes simply dependent on the number of compounds analyzed. The same is true for setting the concentration used for the risk assessment a priori to any other value above zero (Gustavson et al., 2017). The technical annex outlines several statistical methods that can be considered (Technical Annex A, Section 1.2.2). In summary, many of the available statistical techniques dealing with “less-than” values are not fit for data set with >50% of non-detected values. As a result, **sensitivity analysis** was carried using following two scenarios (see technical Annex C) and considering the first scenario being more relevant:

- Assuming zero concentration for all non-detects which is conceptually a best-case scenario but is probably **more realistic scenario** than the next scenario because 1) the high number of non-detects (and the zero-inflation phenomenon, see Technical Annex A, section 1.2.2) especially when expressed as hazard quotients or risk ratios and 2) in general, mixture interactions (including antagonism, potentiation, and synergies) usually occur at medium or high dose levels (relative to the lowest effect levels). At low exposure levels, they are either unlikely to occur or are toxicologically insignificant (SCHER, 2011). And 3) it is widely described in literature that typically only a handful substances contribute to the cumulative risk (Gustavsson et al. 2017, Vallotton et al. 2016, Verro et al. 2009, Posthuma et al. 2016, Backhaus and Karslsson 2014), it is therefore not expected that several non-detected concentrations would have a significant impact in the toxic unit calculation.
- Assuming detection limit as the observed concentration for all non-detects, which is conceptually a worst-case scenario but is most likely an **unrealistic scenario,** especially when detection limit is close to the HC5 or PNEC (Annex A, section 1.2.2, contains an example of generation of artificial risk due to this phenomenon and how this would introduce a bias to the cumulative assessment).

Table 3: Cumulative risks (Hazard Indices: HI) for representative data sets based on lower tier assessment demonstrate that the majority of the observed mixtures in the environment (>80%) are not at risk (for more realistic scenario with respect to non-detects).

	Number of samples/ analytes	% of mixtures not exceeding HI of 1 (HI <1)	% of mixtures exceeding HI of 1 (HI >= 1)	90th percentile and maximum MCR
EU waterbase[a]	12356/	50.8	49.2	2.3/7
EU waterbase without metals[a]	11626/	92.9	7.1	2.4/6
Adour-Garonne (France)	3563/77	93.6	6.4	1.74/3.1
Rhine river	394/51	46.4	53.6	3.0/4.4
Rhine river after refinement[b]	394/51	83.8	16.2	3.0/7.4

[a] *Based on assessment conducted by Rodea-Palomares et al. (in preparation).*
[b] *Refinement was conducted using chronic PNECs derived from the target lipid model for polycyclic aromatic hydrocarbons. Further refinements are likely possible based on toxic mode of action and bio-availability of key contributing metals.*

The cumulative risk analysis showed that the **vast majority of the mixture exposures are not at risk** (i.e., combined risk acceptable HI<1, more realistic scenario). For the Adour-Garonne database, 94% of the mixture samples were predicted not to be at risk. This finding was corroborated by the findings of a recent study for the EU Waterbase dataset (Rodea-Palomares et al., in preparation), for which more than 90% of the samples (without metals) were identified being of no concern. In an initial assessment, cumulative risk was predicted to be substantially higher in the Rhine dataset, with only 46% of the mixture exposures predicted to be not at risk. However, when refining the analysis with more relevant effect levels for PAHs, cumulative risk was predicted to be much lower. After the adjustment, 84% of the mixture exposures were predicted to be not at risk, supporting the conclusions drawn based on the EU Waterbase and Adour-Garonne dataset that overall, the

actual mixtures of potential concern compromise only a small portion of the total mixture exposures. It is assumed that further refinements, e.g., based on toxic mode of action, would further decrease the number of actual mixtures of potential concern. The unrealistic worst-case scenario (assuming non-detects as detects at their detection limit) results in 98% potential risk for the Rhine dataset and 100% potential risk for the Adour-Garonne (France) data set.

The identified drivers of mixtures heavily depend on the monitored chemicals in the database. In the Adour-Garonne region, the following substances contribute dominantly to the cumulative risk: bisphenol A, chloroacetic acid and cyanide, and the following pharmaceuticals: estrone, paracetamol and ibuprofen. Paracetamol, ibuprofen and bisphenol A have been placed on the list of emerging substances of the NORMAN-network. The chemicals that drive cumulative risk in the Rhine river (after refinement of PAH effect levels) are the metals Zinc, Copper and Chromium and the pesticides diazinon and isoproturon. Metals have been noted to be important contributing substances by Price et al. (2012) in a set of different water monitoring datasets and for the Danube. However, it should be noted that none of these references, similar as in our analysis, used bioavailability-corrected site-specific effect levels. It can be expected that predicted metal toxic pressure will decrease when bioavailability corrections are applied (van Regenmortel et al., 2017).

Overall, this is in agreement with observations of the contribution of chemicals across different mixture pressure assessments. **Several studies have listed the same substances as drivers of predicted toxic pressure**:

- Price et al. (2012): bisphenol A, isoproturon, estrone, metals
- Posthuma et al. (2019): bisphenol A
- Bush et al. (2016): isoproturon
- Danube Joint Survey: bisphenol A
- Moshet et al. (2014) diazinon
- Backhaus et al. (2014): metals (Danube),
- Beckers et al. (2018): diazinon (river Holtemme)
- Munz et al. (2017): diclofenac, diazinon, clothianidin (Switzerland)
- Tang et al. (2014) found pharmaceuticals to dominate mixtures in WWTP, in addition to pesticides and other industrial chemicals.
- Rodea-Palomares et al., in preparation: chlorpyrifos, ibuprofen, diazinon, isoproturon, diclofenac

Several of the identified risk driving substances are already identified and managed by specific regulation frameworks;

- Bisphenol A is a Substance of Very High Concern (SVHC), is included in the candidate list for authorization and was ranked high under the WFD prioritization list.
- Chloroacetic acid was placed in 2005 on the priority list under the Existing Substance Regulation
- Estrone has been placed on the WFD watch list (EC 2018/840)

- Pharmaceuticals: the European Commission has set out a strategic approach in relation to the risks from pharmaceuticals in the environment (EC 2019)
- Isoproturon and diazinon are no longer approved in the European Union (EC 91/414/EEC)
- Chromium has been shortlisted for the WFD priorisation list (Negrao Carvalho et al. 2016)

Apart from the identified risk driving substances, the identification of the many non-risk driving substances is equally relevant mentioning.

Van de Meent et al. (2020) calculated the relative contributions of the different chemicals to the mixture toxic pressure at the EC50 level, and concluded that, for each group of chemicals (REACH substances, pharmaceuticals, and pesticides), a relatively small fraction of the chemicals was clearly responsible for a large fraction of the toxic pressure. In line with this, Rodea-Palomares et al. (in preparation) demonstrated for the EU Waterbase that unmeasured risk is likely captured by existing monitoring programs. The hazard index for mixtures increases as the number of monitored chemicals increases until a plateau is reached for the hazard index where additional monitored chemicals would not lead to an increased hazard index. Similar observations have been made in other fields of science (Muller, 2016) and have been termed the 80–20 rule, or (more correctly), the **Pareto principle**.

Several chemicals can be regulated under different regimes (e.g., REACH, biocides, plant protection products), can also be secondary pollutants like combustion products (e.g., PAH) or can originate from historical emission sources. This whole analysis confirms that mainly so-called known legacy chemicals (and uses) drive the current observed mixture toxic pressure. This is not unexpected because priority pollutants have been identified as hazardous for the environment and have been monitored extensively in the EU. This observation is further corroborated by the analysis of Rodea-Palomares et al. (in preparation) which showed that mixture risks is mainly driven by priority chemicals, while the risks imposed by other measured substances is small.

Naturally occurring substances, such as metals and natural hormones, will even in pristine situations result in a geochemical or biological **background concentration**. It has been argued that risk evaluation methods, such as the HI/HQ approach, are not suitable for naturally occurring substances, as they might predict risks below natural background concentrations (e.g., metals: Van Regenmortel et al. 2017, Nys et al. 2018). This is further complicated in the case of essential elements for which regulatory limits might be set in the range where deficiency occurs (Meyer et al. 2015). In several regulatory applications for metals, background metal concentrations have been derived as the 10^{th} percentile of metal concentrations from the geochemical baseline dataset representing pristine freshwater streams published by the Forum of European Geological Surveys (FOREGS, Salminen et al. 2005). The Hazard Index associated with these background concentrations of metals in European waters is 1.4, indicating that even at background concentrations of metals the HI/HQ approach calculates unacceptable risks of mixture exposure. For metals, bioavailability is essential and physico-chemistry parameters such as dissolved organic carbon, pH and hardness substantially influence toxicity (Mebane et al. 2020) and therefore also environmental threshold levels (e.g., Van Sprang et al. 2016).

Not accounting for bioavailability in metal risk assessment approaches leads inevitably to overestimation of risks. Hence, bioavailability models, such as biotic ligand models are currently widely used in environmental risk assessment and EQS compliance checking. However, even when accounting for bioavailability, Nys et al. (2018) showed that 13% of water samples from a database representing geochemical baseline concentrations in pristine European waters (i.e., the FOREGS database) are considered to be at risk using the HI/HQ approach (i.e., HI>1) when only the following 5 metals are considered: Zn, Ni, Cu, Cd and Pb. With the HI/HQ approach, naturally occurring substances will always contribute to the risk, as the HQ is only zero if the concentration is zero. Hence, for naturally occurring substances the HI/HQ approach is often unrealistic. This is especially the case for naturally occurring substances that are essential for life, where the boundary between essentiality and toxicity is narrow and the HI/HQ approach might predict risks in the zone where essentiality occurs. This demonstrates the overall approach (both for essential natural as well as man-made substances) is overpredictive and conservative. As an approach to deal with naturally occurring substances such as metals, the **added concentration approach** has been used. The added approach assumes that species are fully adapted to the natural background concentration and therefore the natural background concentration does not contribute to the toxicity. This approach accounts for background concentrations of naturally occurring substances by subtracting the background from the measured environmental concentration.

The overall conclusion is that the **majority of observed monitored mixtures is predicted not to pose a potential concern** (84-94% of the samples were predicted being of no concern, even when a conservative approach of additivity is assumed). **For those mixtures of potential concern, several of the identified risk driving substances are already identified and managed by specific regulation frameworks.** These risk driving substances in mixtures include a combination of agrochemicals, pharmaceutical, industrial and household chemicals. Taking into account toxic mode of action could further reduce the number of mixtures of potential concern by 75-85%. The **use of the MAF needs therefore to be differentiated (relevant for risk driving substances in mixtures with potential concern and not managed by existing regulation frameworks) and proportional** to the magnitude of the mixture toxicity problem.

2.4. IDENTIFICATION OF MIXTURES WITH PREDICTED CUMULATIVE RISK

Next, **identified mixtures of concern in hotspots** are **further assessed**. Hotspots can be divided in locations with co-exposure due to the presence of several substances exceeding their threshold or where co-exposures of potential concern are identified in the absence of clear risk drivers. Here, we try to identify possible common use patterns that result in co-exposures of concern. The ultimate aim is to **identify co-exposures of potential concern that would not be identified by a single substance assessment.**

Price et al. (2012) described a methodology to group mixture exposures into different categories to aid the evaluation of mixtures of concern and support risk management decisions. The purpose of the Cefic MIAT

approach is to identify potential co-exposure of concern. This methodology differentiates combined exposures based on an assessment of the HQ_i, HI and MCR (see Section 2.3 for definitions). In practice, four exposure groups are distinguished.

- **Group I:** Combined exposures that are a concern because one or more individual chemicals are a concern: i.e., at least one of the HQ_i>1; this situation can be identified with existing chemical-by-chemical RA.
- **Group II:** Combined exposures where there is a low concern for both individual chemicals and for their combined effects: i.e., HI<1
- **Group IIIa:** combined exposures where there is a low concern for individual chemicals but there is a potential concern for the combined effects; one chemical provides the majority of toxicity of the combined exposures: i.e., all HQ_i<1, HI>1 and MCR<2
- **Group IIIb:** combined exposures where there is a low concern for individual chemicals but there is a potential concern for the combined effects; no one chemical is dominating, i.e., all HQ_i<1, HI>1 and MCR>2

The spread of the mixture exposures over the different mixture groups for the analyzed datasets is shown in Figure 9. The risk driving substances are further detailed in Annex C). In practice, the individual potential risks in Group I (HQ > 1) are regulated by single-substance regulations. For Group II mixtures, no potential risk is identified, and these mixtures are considered to be of 'low concern'. Group III mixtures are the focus of interest of a cumulative risk assessment. This is because the cumulative risk observed in these mixtures are not managed in current single substance legislation. Group IIIa mixtures can be remediated by targeting the substance that is responsible for the majority of the cumulative risk. Group IIIb represent the mixtures where none of the constituents is dominating the predicted risks, which should be further addressed by incorporating mode of action.

Overall, the **Group III mixtures represent only a minority of all observed mixture exposures** (see Table 4). In the Adour-Garonne database, less than 1% of the mixture exposures are categorized as either of the Group III mixtures. For the Rhine river in the initial assessment, this picture was quite different with 36% of the mixture exposures categorized as Group III (see Table 4). The Rhine data set should be considered as worst-case because there are only few monitoring locations and these monitoring locations represent hotspots since there are mostly located on the main Rhine river (no locations on major or smaller tributaries) and near the downstream part of the river basin accumulating emissions from the whole Rhine catchment. However, refining the analysis for the PAHs (see above) already resulted in significantly fewer complex mixtures that were identified (i.e., 12.6% Group III mixtures). As metals are some of the main drivers in the Rhine database, it can be expected that bioavailability corrections and the added concentration approach for metals would further improve the identification of mixtures of concern.

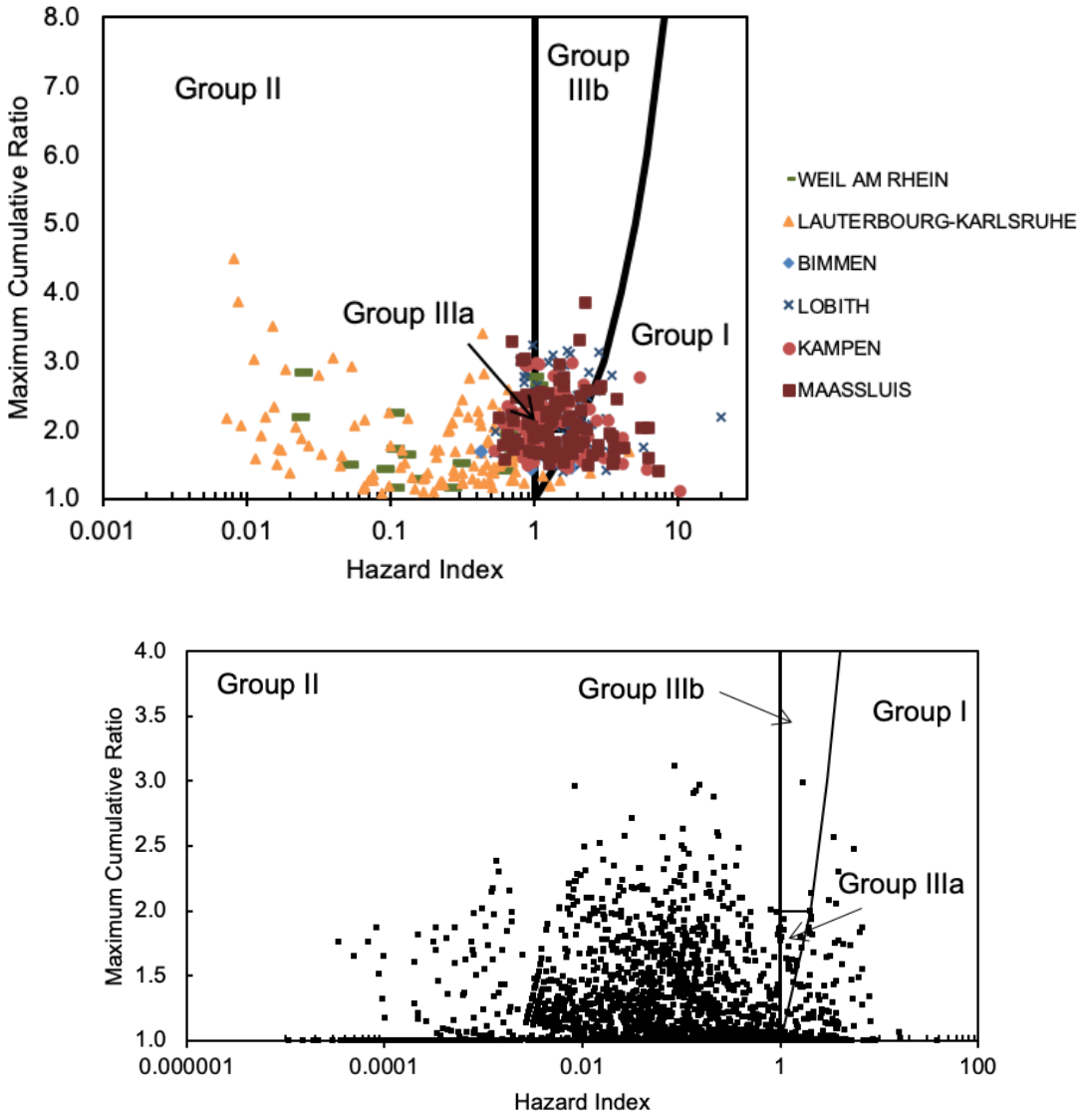

Figure 9 Plot showing the Maximum Cumulative Ratio as a function of the Hazard Index for the Rhine river (upper panel, different symbols represent different sampling sites) and the Adour-Garonne dataset (lower panel) for the more realistic scenario (showing cumulative risks of the detects only).

The low percentage of mixture exposures identified as complex mixtures is further corroborated by the assessment based on the EU Waterbase dataset, where 9% of the samples were identified as Group III mixtures (Rodea-Palomares et al., in preparation). Further support for this observation is also given based on a set of freshwater monitoring databases from the EU by Price et al. (2012; 12% of Group III mixtures), and from the US by Vallotton and Price (2016, 7.7% of Group III mixtures). However, these last two assessments are largely based on acute effect levels.

Table 4: Percent of mixture exposures that fall into the four mixture groupings of the CEFIC-MIAT decisions tree for selected databases (without further refinements except for one refinement in the Rhine basin) (more realistic scenario with respect to non-detects).

	Group I Concern because of individual risk	Group II Low concern	Group IIIa: Concern for combined effects, one chemical dominates	Group IIIb: Concern for combined effects, no one chemical dominates
EU waterbase after excluding metals	3.7%	92.9%	1.1%	2.4%
Adour-Garonne (France)	5.8%	93.6%	0.5%	0.1%
Rhine river after first refinement	3.6%	83.8%	2.5%	10.1%

The percentage of mixtures with potential concern not identified by single substance assessment is small (<=13%) even without further refining (e.g., by looking into toxic mode of action) and are spatially spread across EU Member States. The risk driving substances can have varying physico-chemical properties (organics versus inorganics versus PAH), can have varying use and temporal patterns and are regulated by different legislations (agrochemicals, pharmaceuticals, industrial and household chemicals). It may therefore be more effective to consider risk management a level integrating multiple substances and uses. Applying a straight MAF in all single substance assessment impacts all groups and may not be sufficiently targeted to the issue. The **use of the MAF needs to be differentiated (relevant for mixtures at potential concern) and proportional** to the magnitude of the mixture toxicity problem.

2.5. UNDERSTANDING MIXTURES IMPACT

The datasets were screened regarding availability of relevant and reliable ecological endpoints and / or bioassay effect levels for the same location / samples. Biology, chemicals and water chemistry are often part of different monitoring programs and not collected with an integrated analysis in mind. Therefore, it was a **challenge to link biology with relevant chemical exposure**, limiting the datasets and the type of analysis possible (Technical Annex D section 4.1). Only for the Adour-Garonne and the Flanders dataset a comparison between biological status and chemical exposure was possible.

For the Adour-Garonne dataset, a negative (statistical) effect of the summed toxic units of measured chemicals on biodiversity indices was found, suggesting that chemical pressure negatively affects biodiversity. It should be noted, however, that this effect was small compared to effects found for water chemistry parameters i.e., four times lower than the effect of organic nitrogen (Kjeldahl), half as strong as the effect of conductivity and similar in magnitude to total phosphorous. The relative importance of chemical exposure was thus low compared to other water chemistry parameters, especially nutrients. Additionally, this only expressed the influence of chemical exposure as a whole (summed toxic units) and does not allow to differentiate between

effects of single exposure and co-exposure. The strong correlation (0.99) between maximum toxic unit and summed toxic units indicates that the chemical pressure was dominated by one chemical and not by co-exposure of chemicals. For Flanders, mixture toxicity from metals had a negligible influence compared to the water chemistry, especially nutrients, while pesticides seemed to have a larger effect, although it should be stressed that the latter analysis was performed on a limited dataset.

In general, **strong conclusions on the biological impact of mixtures could not be drawn based on the statistical analyses in this project**. Coupling the chemical and biological monitoring data was an issue and should be tackled more thoroughly e.g., by aggregating data based on spatial coordinates or gaining access to the raw data. The analyses we were able to perform suggest that chemicals can have a negative association with measured biodiversity indices but that other environmental factors, mainly nutrients, are more critical to determine the biological status of surface waters. Additionally, we were not able to discern the biological impact of single chemicals from mixture effects, but the analysis for the Adour-Garonne dataset suggested that biological effects were most likely cause by single chemicals rather than the combined effects of multiple chemicals.

3. HUMAN HEALTH

Throughout their lifetime, humans are exposed to a mixture of environmental stressors and chemicals that independently or in interaction may have an impact on health. These mixtures of exposure can consist of an almost infinite number of different combinations of chemicals, which makes the exposure and risk assessment challenging. A detailed assessment of risks for all possible unintentional mixtures under REACH and other regulatory sectors seems unattainable, due to the required knowledge about emission and exposure sources and the large number of exposure assessments.

The European Commission recognizes that consideration of unintentional mixtures is broadly absent in legislation oriented towards chemicals and described recently a possible ways forward to address challenges in the regulatory context. The Commission considers the Mixture Assessment Factor (MAF) as a pragmatic approach to mixture assessment, in order to manage the current situation of lack of the necessary toxicity and exposure data and make a systematic regulatory management of unintentional mixtures possible in a relatively near future.

By preference, the magnitude of the MAF should be supported by scientific data. In relation to the MAF discussion for human health, the use of human biomonitoring (HBM) data can be considered since this type of data forms a good reflection of real-life human exposure since HBM data reflect exposures from all routes and sources, and in nearly all HBM studies, exposure to multiple chemicals has been monitored (albeit rather few chemicals).

In this study, an overview of existing HBM datasets was made, and the relevance and potential use for human health mixtures risk assessment was assessed.

3.1. DESCRIPTION OF EXISTING HBM DATASETS

The Horizon 2020 project 'Human Biomonitoring for Europe – science and policy for a healthy future' (HBM4EU; www.hbm4eu.eu) generated an extensive overview of biomonitoring datasets in the EU. There is a wealth of existing HBM datasets (>100) described in the EU, with potential relevance for mixtures in relation to human health risk assessment. For the majority of the studies (fulfilling criteria such as minimal sample size of 50) aggregated data are accessible via the HBM4EU dashboard (https://www.hbm4eu.eu/eu-hbm-dashboard/). These include summary statistics, such as the percentiles of the biomarker levels per data collection for 18 HBM4EU priority substances or substance groups (acrylamide, anilines, aprotic solvents, arsenic, bisphenols, cadmium, chromium, flame retardants, lead, mercury and its organic compounds, mycotoxins, per/polyfluorinated compounds (PFAS), pesticides, phthalates and DINCH, polycyclic aromatic hydrocarbons (PAHs) and UV filters (benzophenones)).

On the one hand, it is recognized that the majority of HBM studies (18 chemical classes, covering 152 biomarkers of exposure) cover only a small subset of the 'real world mixtures', in view of the thousands of chemicals produced by industry and even more natural chemicals often ingested in much higher doses, as well as pharmaceuticals. On the other hand, these 18 substance groups should not be minimized too much, since the selection of these groups was a result of a thorough HBM4EU prioritization strategy, considering relevance for human health.

Some of the chemicals included in the HBM campaigns pertain to legacy chemicals (e.g., hexabromocyclododecane 'HBCDD', Hexachloro-1,3-butadiene 'HCBD', PFOA and PFOS) or chemicals with restrictions (e.g., DEHP, bisphenol A) and are therefore rather a reflection of past exposure, in combination with current exposure to substances present in materials with long lasting uses (e.g., furniture, construction products). The consideration of legacy chemicals in the MAF discussion should therefore be considered.

Recent small pilot studies using suspect and non-target screening on human biomonitoring data also demonstrated that the 'classical' targeted screening (such as biomarkers belonging to the 18 HBM4U priority groups) does not capture a substantial number of industrial chemicals present in human body fluid, and thus the mixtures that are identified using target screening are very likely an underestimate of the real mixtures in human biofluids. The field of suspect and non-target screening is an upcoming field, and once it will be enrolled in several HBM studies (as anticipated under the PARC initiative[1]), it can be very informative to inform on composition of mixtures in HBM samples (Pourchet et al., 2020).

The currently available HBM datasets in EU can be seen as a 'patchwork' of studies, from different regions, with each study having a particular scope and research question, and therefore different population groups (age/gender), periods and different sets of chemicals monitored. The HBM4EU project puts strong efforts in setting up aligned studies, which will enable in the future a better comparison of results across studies. The observation of diversity in HBM studies in several aspects should not hamper the use of individual datasets for mixture risk assessment; though comparison of risk estimates as outcomes of different studies should be interpreted with caution, and accounting for the different characteristics of the studies.

Investigating mixture patterns on human biomonitoring datasets and performing mixtures risk assessment requires access to individual datapoints. Aggregated datasets are useful to perform single substance risk assessment. Aggregated datasets entail information for various single chemicals; however, the aggregated level does allow insight in co-occurrence patterns or dependency of distributions patterns of single chemicals. Attempts have been made to use aggregated datasets to create mixtures scenarios, and related mixtures risk assessments (see further). However, the representativeness of such 'constructed scenarios' in view of realistic mixtures is questionable. The ability to get access to (pseudonimized) individual HBM datasets is much more difficult compared to individual datasets from environmental monitoring campaigns, given the confidentially and privacy concerns of individual's health data. Thus, information at the individual level is not publicly available

[1] PARC = Partnership for the Assessment of Risk from Chemicals (launched under the Horizon Europe Programma)

for any human biomonitoring dataset. Access to this type of data might be requested upon a motivated request submitted to the data owners/controllers of the datasets (the duration of the procedure and conditions to get access currently differ from study to study). Improvements of data sharing of individual records to perform mixtures risks assessment is essential to move forward with human biomonitoring mixture risk assessment (Bopp S, 2016).

Despite the large number of humane biomonitoring (HBM) studies, the analysis of mixtures co-occurrence patterns in HBM data, observational studies linking mixtures to health outcomes, and mixtures risks assessments, seem to be less covered in the scientific literature compared to single substance risk assessments and health associations.

3.2. EVIDENCE ON CO-OCCURRENCE OF CHEMICALS IN HUMAN BIOFLUIDS

3.2.1. CO-OCCURRENCE IN VIEW OF SIMULTANEOUS PRESENCE/ DETECTION OF CHEMICALS IN HUMAN BIOFLUIDS

Exploring the HBM4EU dashboard, we noticed that for a first group of chemicals (including several of the 18 HBM4EU priority chemicals or chemical groups), at least one exposure biomarker has been detected in urine or blood in nearly all available samples of general populations (i.e., measurements above LOQ/LOD in more than 95% of the investigated samples). This is the case for acrylamide, anilines, arsenic, bisphenol A, cadmium, chromium, lead, mercury, PFOS and PFOA, some pesticides (3-PBA; glyphosate), phthalates and PAHs. It should be remarked that not all these chemicals are systematically monitored in all datasets, but *if* monitored, it appears that they have a high detection frequency.

There is a second group of chemicals which are present in a part of the population (e.g., DINCH, some organophosphate pesticides, several PFAS: present in > 50 % of samples if LOQ is sufficiently low), they were not detected in another part of the population. For a thirds, smaller set of chemicals, levels are only detected in a small proportion of the datasets (i.e., P90 or above > LOQ/LOD); e.g., this is the case for benzophenones (UV filters), some pyrethroids pesticides and some BDE's. For the group of BDEs: there is a rather diverse pattern: BDE-153 is detected in the majority of samples (P5 > LOQ) in some databases, while having a smaller detection rate in other databases. Other chemicals belonging to the BDEs are only detected in the minority of the samples of some datasets (e.g., BDE 183).

The detection frequency cannot be fully compared across different studies, since LOQ and LODs might differ across studies (up to 20-fold or more), explaining partly why in some studies a higher proportion of the samples is < LOD/LOQ. Besides the difference in LOQ and LODs across datasets, also other factors play a role in detection frequencies across datasets e.g., age of the study participants, sampling years, the region of the study and the specific population. For example, DINCH metabolites were only detected in 25 % of urine

samples the ESB_2009 study in Germany in 2009; while keeping the same LOQ, DINCH metabolites became ubiquitous in the samples in the next decade (up to presence in > 95%), likely due to the increased use of DINCH as a substitute for DEHP.

In summary, in the majority of the human biofluids in HBM campaigns, a multitude of chemicals have been detected, thus providing evidence for simultaneous exposure to several chemicals.

3.2.2. CO-OCCURRENCE IN VIEW OF MAGNITUDE OF EXPOSURE (CONCENTRATION LEVELS) OF CHEMICALS IN HUMAN BIOFLUIDS

For several HBM studies, co-occurrence patterns of chemicals in human biomonitoring datasets in ***terms of magnitude of exposure (concentration levels)*** have been reported. The co-occurrence patterns in these studies are based on a variety of statistical techniques including linear regression, heat maps, circular plots, principal component analysis (PCA) and network analysis. Given the diversity of the studies (population group, sampling years and monitored chemicals) conclusions differed from study to study. Overarching over different studies, the following trends and observations were made: ***Mainly levels of chemicals belonging to the same chemical group tend to correlate.*** For example, samples with high levels for one PCB, have a likelihood to have also high level of other PCBs, and vice versa (this was demonstrated in several HBM Studies, Rosofsky et al., 2017; Govarts et al., 2020; Ottenbros; Agay-Shay et al., 2015; Tamayo-Uria et al., 2019).

Co-occurrence of magnitude levels between several PCBs can be explained by the presence of several PCBs in technical mixtures, thus PCBs having a common source. Likewise, levels ***within*** the groups of PBDEs, PFAS, phthalates, metals, phenols and pesticides tend to be correlated. Co-occurrence of elevated levels across chemical classes is in general weak or not present unless for chemicals occurring in or originating from the same sources. For example: co-occurrence of elevated levels PCBs and Hg can be explained by fish as common source and co-occurrence of mono-ethyl phthalates and parabens was explained by personal care products as common source (Rosofsky et al., 2017).

A lack of a co-occurrence patterns (mainly across chemical groups) based on ***levels*** should not be interpreted as that the ***presence*** of those chemicals is mutually exclusive. Rather, it indicates that chemicals with lack of co-occurrence patterns (levels) are “randomly” co-occurring. Let’s say if chemical A and B are both detected in the entire population, but they lack a co-occurrence pattern based on *levels,* it means that they both occur in every sample in the population but the concentration levels of A and B are independent of each other. A lack of co-occurrence regarding levels in humans can also be regarded as substances having independent distributions.

When performing human health risk assessment on datasets at individual level (cf. recommended approach), there is no need to explicitly account for co-occurrence patterns, since this type of information is inherently present in databases. However, such information would be valuable for investigations into what use types or

substance properties increase the likelihood of combined exposures, so that improved exposure models for combinations can be developed. When one wants to perform mixtures risk assessment on aggregated data (not the recommended approach), the dependency of distributions should be considered, for chemicals with co-occurrence patterns based on levels.

3.2.3. MIXTURES IN RELATION TO HEALTH EFFECTS

Several studies performed in the EU regarding mixtures in human biomonitoring data investigated the relation between mixtures exposures and health outcomes. The majority of these studies focus on perinatal exposure (exposure assessment in cord blood of newborns, breastmilk, urine, blood/serum of pregnant mothers) in relation to perinatal health outcomes (birth weight, placental weight, birth length, head circumference), and sometimes health outcomes in the follow-up study (e.g., BMI at 7 years; early menarche of female offspring).

Statistically significant associations between mixtures exposure (from nowadays levels of chemicals present in humans assessed by human biomonitoring) and health effects in the (vulnerable groups of) general population have been reported in several studies. Not all effects were observed using single chemical models, and associations were stronger when mixtures were considered compared to single chemical exposure associations. Current exposure levels, for example PCBs, in human bodies were often caused by historical contamination, but are still found in human samples due to the persistency and long half-lives.

While some studies reported associations between mixtures exposure and adverse health outcomes, there were also some studies showing presence of mixtures in biofluids, where no adverse effects were observed. An overarching conclusion over different studies is difficult to draw given the differences in characteristics in chemical mixtures, different methodologies and different outcomes and different cohorts investigated across studies

The observation that some epidemiological studies on specific groups of similar chemicals show associations correlation with adverse effects due to mixture exposure shows a need to better understand the role of combined exposure in the context of human health risk assessment.

It's important to realize that the **current epidemiological associations** on mixtures in relation to health effects is mainly focused on a **specific, vulnerable subpopulation** (newborns), considering **generic health outcomes** and a **limited list of chemicals** (including legacy chemicals, and chemicals under restrictions). Epidemiological studies considering effects of mixtures in other vulnerable populations and life stages (e.g., puberty, elderly) and other, more specific categories of health effects (e.g., effects of mixtures on neurodevelopmental effects, cardiovascular effects, respiratory system, immunological effects, etc.) remain to be investigated. Additionally, the associations between health outcomes and mixtures pertain often to legacy chemicals, which have been used in the past, but are currently no longer used or limited in current applications

(e.g. POPS under Stockholm Convention, chemicals under Restriction or Authorization). In view of the discussion of MAF for REACH chemicals, it would be very useful to have this type of analysis performed also on mixtures of non-legacy chemicals.

3.2.4. MIXTURE RISK ASSESSMENTS USING HBM DATASETS

The statistical models described in above mentioned epidemiological studies cannot be used in regulatory risk assessment concepts such as RCRs under REACH. Instead, a practical instrument to perform mixtures risk assessment is the **Hazard Index (HI)** approach, in combination with **maximum cumulative ratio (MCR)**. The HI of a sample (biofluid sample) is the sum of hazard quotients (HQ) of several chemicals present in the biofluid. In its turn, the HQ is the ratio of the concentration (exposure level) to the health-based guidance value per substance. The MCR is the ratio of the HI of the mixture to the maximum of the HQs of the individual components, and thus represents whether the risk is mainly driven by one substance (MCR =1), a few substances (MCR: 2-3), or caused by a lot of substances all contributing a little bit to the risk (MCR → n; with n = number of substances present in the mixture). The HI and MCR approaches are based on the hypothesis of dose addition, which is considered a conservative assumption for evaluating mixture effects, especially when applied in a low tier approach without considering communalities in endpoints or mode of action, and thus including all substances in the HI summation. The MCR and HI approach can also be used in a higher tier approach, where the summation of HI considers only chemicals affecting the same health effect or according the same mode of action.

In several publications, the HI and MCR concept have been applied in human biomonitoring data. Nearly all studies calculated HI and MCR on individual datapoints in datasets and relied on reverse dosimetry of HBM data (urinary levels) to external exposure estimates, which are used in HI calculations, in combination with health-based guidance values for external exposure for single chemicals. Nearly all publications deal with mixture risk assessment covering risk of substances *within a chemical group*; hardly any of the publications calculated risks across chemical groups.

The majority of the HBM HI/MCR studies within chemical groups pertain to mixture risk assessment for **phthalates**, with a focus on risk for reproductive and developmental toxicity by anti-androgenic modes of action, since this is the critical and common health effect of several phthalates. To varying degrees, these studies revealed exceedances both of acceptable single and combined phthalate exposures, and in general the HI was dominated by one or two phthalates (dominance of DBP and DEHP in the HI). However, the outcomes of these various phthalate mixture risk assessments are difficult to compare because of three reasons. Firstly, different phthalates and numbers of phthalates were investigated in the different studies. Secondly, the studies used often different health-based guidance values (EFSA 2005 TDIs for phthalates versus RfD for phthalates derived by Kortenkamp & Faust, 2010 and Kortenkamp & Koch, 2020). This factor

might lead to a 5-fold difference in HI values, if all other factors are kept constant. Thirdly, there is also evidence that phthalate exposures have undergone changes over the years. Apel et al. (2020) performed HI and MCR calculations for each participant from the German Environmental Specimen Bank, which is a 27-year survey of urinary phthalate metabolite levels in 24-hour urine samples. While (Apel et al., 2020) reported strong (about 10-fold) declining HI's over this period, ascribed to decreasing exposure levels, they reported at the same time that the more recent HIs were driven by a greater number of phthalates..

Additionally, studies on application of HI and MCR concept on human biomonitoring data were found for parabens (Moos et al., 2017), dioxins, furans and PCBs (Han and Price, 2013), pesticides (F. Fernández et al., 2020; Katsikantami et al., 2019) and PFAS (Borg et al., 2013). Also, for these chemical groups, one or a few compounds dominated in general the HI (i.e., n-PrP was the dominant substance in the HI for parabens; for PCBs: MCR: 2-5; for pesticides: dominance of dimethoate in the organophosphate group; dominance of PFOS and PFOA for respectively the HI hepatoxicity and reproductive effects). However, since PFOA and PFOS are phasing out, the use of other PFAS is increasing, which is visible in more recent HBM studies. Therefore, the pattern of dominance of PFOS and PFOA in the mixture toxicity is likely to decrease, and other PFAS might play a dominant role in the HI in the future.

A few studies have looked at the HI **across different chemical groups**. For example, the human health mixture risk assessment of (Kortenkamp and Faust, 2010) included exposures to a combination of 15 chemicals from several groups: phthalates (DBP, BiBP, BBP, DiNP, DEHP), pesticides (vinclozolin, prochloraz, procymidone, linuron, fenitrothion, pp'DDE), a flame retardant (BDE-99), bisphenol A, and parabens (butyl paraben and propyl paraben), all being substances capable of producing reproductive and developmental toxicity by anti-androgenic modes of action. Based on their data, we estimated MCR values of 2.0 and 3.2 for the median and high intake scenario, respectively. On the one hand, this study had an important shortcoming in the sense that the exposure assessment is based on aggregated data from a variety of sources, and not on individual data. Hence, it is unknown if indeed all individuals were exposed to the assumed mixtures, or to which levels of the different chemicals. On the other hand, the concept of applying HI and MCR calculations on substances across chemical groups considering common health effects, is interesting. It would be useful to apply their concept of across chemicals groups mixtures risk assessment on individual exposure data records instead of aggregated data.

Several authors point to consider mixtures risk across chemical groups ((Borg et al., 2013; Kortenkamp, 2020)) in order not to miss part of the mixture toxicity. However, **a 'blind' summing is not recommended for mixture risk assessment on HBM data**. To proceed, further developments should be made in view of gathering detailed toxicological information for grouping, point of departure values for relevant endpoints, and adverse outcome pathways, and derivation of endpoint specific guidance values. A discussion of the selection of the health-based guidance values as denominator in the HQ could also be welcome, since this can affect strongly the HI values. This was noted when using Reference Doses (RfD's from EFSA for phthalates versus guidance values derived by Kortenkamp et al. 2010 and 2020. For many chemicals, the **choice of the health-based**

reference values is likely to have a strong impact on the mixture risk. This discussion should also consider the role of human biomonitoring guidance values (HBM-GV) as denominator in the HI. Using HBM-GV would allow a straightforward use of HBM exposure levels (in urine, blood), instead of back-calculated external exposure using reverse dosimetry or empirical factors. The latter introduce uncertainty in the exposure assessment.

In the majority of the investigated studies, a few compounds drive the toxicity (MCRs typically 2-3). However, two important considerations should be made: this conclusion is often based on results from within group chemicals mixtures risk assessment, with a limited number of substances considered (e.g; phthalates: often only 4-6 compounds considered). It should be further investigated whether this dominance of a few compounds is still valid when considering across chemical groups risk assessment (thus including substantially more chemicals) and considering time trends where substitution towards more compounds (e.g., PFAS and phthalates) is likely to occur.

As final reflection: mixture risk assessments rely on the assumption of the dose additivity concept and do not consider other possible types of interactions (e.g., synergism or antagonism) that are not considered to occur widely but that might occur for specific combinations. For a practical point of view, it is not feasible to consider other types of interactions in regulatory mixture RA).

4. GENERAL CONCLUSIONS

Environment

This report is trying to understand how chemicals co-occur, and which combinations dominate and may potentially present a concern. This will be the starting point for appropriate identification of uses, locations or types of chemicals which should be managed by application of a MAF. Starting from a wide array of spatially broad monitoring databases allows a unique and realistic assessment of chemical co-exposure over a wide range of conditions:

- The majority of the chemicals are not detected. Considering all databases, typical detected co-exposure/mixtures consist of a combination of agrochemicals, industrial, household and pharmaceuticals. Also, risk driving substances in mixtures include a combination of agrochemicals, pharmaceutical, industrial and household chemicals. This may lead to the conclusion that implementation of a MAF is to be considered in a broader perspective t
- han the REACH Regulation alone.
- Co-exposure is dependent on the location and time of monitoring, which are in turn an indication of the underlying use and emission pattern of the chemicals and different management options. The complexity of the mixtures is largely driven by the spatial location, the intensity of anthropogenic activity (household, industry, agriculture) and time. The more downstream a river catchment, the more complex mixtures can be expected both in terms of number of chemicals as well as types of chemicals. Reversely, more simple mixtures can be expected more upstream river catchments and with higher fluctuation of concentrations. The REACH Regulation largely relies on so-called generic exposure scenarios leaving out spatial and temporal character of environmental exposure. This complicates the refinement options in higher tier approaches following application of a generic MAF factor under the REACH Regulation.
- A relatively small fraction of the chemicals is typically found responsible for a large fraction of the toxic pressure. Similar observations have been made in other fields of science (Muller, 2016) and have been termed the 80–20 rule, or (more correctly), the Pareto principle.
- Naturally occurring substances, such as metals and natural hormones, will even in pristine situations result in a geochemical or biological background concentration. Risk evaluation methods, such as the HI/HQ approach without correction for natural background, are not suitable for naturally occurring substances, as they might predict mixture risks below or at natural background concentrations.
- The majority of observed monitored mixtures is predicted not to pose any potential concern (80-94% of the samples were predicted being of no concern, even when a conservative approach of additivity is assumed). The percentage of mixtures with potential concern not identified by single substance assessment is small (<=13% for Rhine dataset, <=2.5% for EU wide databases) even without further refining (e.g., by looking into toxic mode of action). Taking into account toxic mode of action could further reduce the number of mixtures of potential concern by 75-85% and this refinement is spatially spread across EU Member States.

- For those mixtures of potential concern, several of the identified risk driving substances are already identified and managed by specific regulation frameworks. The limited number of risk-driving substances can have varying physico-chemical properties (organics versus inorganics versus PAH), can have varying use patterns and are regulated by different legislations (agrochemicals, pharmaceuticals, industrial and household chemicals) or are legacy substances. Risk management would require an integrative assessment of multiple substances and uses. Applying a straight MAF in all single substance assessment impacts all chemicals and may not be sufficiently and effectively targeted to the mixtures of potential concern. The use of the MAF needs to be differentiated (relevant for risk driving substances in mixtures at risk and not already managed by existing regulation frameworks) and proportional to the magnitude of the mixture toxicity problem.
- Preliminary data-driven possibilities to quantify the MAF to assess the combined toxicity of chemicals in the environment and the humans is evaluated here and in literature (Kemi, 2021). Further research is recommended to further review and compare these approaches on spatially broad datasets. Several improvements can be made on the use and the derivation of the threshold (HC5) that will significantly impact the MAF.

Human

- Despite the large number of human biomonitoring (HBM) studies, the analysis of mixtures co-occurrence patterns in HBM data, observational studies linking mixtures to health outcomes, and mixtures risks assessments, seem to be less covered in the scientific literature compared to single substance risk assessments and health associations.
- A lack of co-occurrence patterns (mainly across chemical groups and between age groups) based on ***levels*** should not be necessarily interpreted as that the ***presence*** of those chemicals is mutually exclusive. Rather, it indicates that chemicals with lack of co-occurrence patterns (levels) may or may not be “randomly” co-occurring. A lack of co-occurrence regarding levels in humans can also be regarded as substances having independent distributions.
- It is noted that a lot of human biomonitoring research focused on legacy chemicals with long half-lives such as PCB’s.
- The observation that some **epidemiological studies on specific groups of similar chemicals exist and do demonstrate an association between health effects and mixture exposure** illustrates the need for further ‘mixture studies’ in human risk assessment. However, this does not suggest that a MAF would be an appropriate tool for such risk assessment. At the same time, it’s important to realize that the **current epidemiological knowledge** on mixtures in relation to health effects is mainly focused on a **specific, vulnerable subpopulation** (newborns), considering **generic health outcomes** and a **limited list of chemicals** (including legacy chemicals, and chemicals under restrictions).
- **In the majority of the investigated studies, a few compounds drive the risk** (MCRs typically 2-3). Additionally, it should be noted that often a limited number of substances are considered and usually

based on results from within group chemicals mixtures risk assessment. Advancements in chemical analysis of human samples using untargeted approach analysis will likely reveal more chemicals and contribute to a better insight in real mixtures in human samples.

- Mixture risk assessments rely on the assumption of the dose additivity concept. In general, additive models are a good approximation, but it should be realized that in some specific combinations other possible types of interactions may occur (synergistic or antagonistic effects). For a practical point of view, it is not feasible to consider other types of interactions in regulatory mixture RA approaches.
- For future research, it is imperative that anonymized human biomonitoring data are available at an individual sample level. Aggregate data do not inform about how many persons out of the study were actually exposed to which chemicals at which levels in which combinations.

Important conclusions on co-exposure for both environment and human

- Dose/concentration addition is typically used as a first-tier approach to mixture toxicity. This approach ignores different modes of actions and temporal exposure and thus does not necessarily reflect the real mixture risk. This should be considered when interpreting predictions of this conservative approach.
- Choice of reference value/effect concentration is critical. Different options are available and the implications should be considered when interpreting the mixture risk predictions.
- A limited number of chemicals tends to dominate the risk profile of mixtures at risk.

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