



# Rapid evidence review – the biological effects of chemical mixtures in aquatic environments

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## Executive summary

The chemical quality of sea, estuaries, lakes and rivers is a topic of high public, media and political interest. Multiple point and diffuse sources emit chemicals to marine and freshwater ecosystems resulting in the widespread presence of chemical mixtures. Research on the exposure, hazards and risks of chemical mixtures to aquatic life is a mature field in both the regulatory and academic sphere. With large-scale water chemistry monitoring data publicly available and 1000s of papers published in the field it is possible to develop and test general principles and models for mixture exposure, effect and risk assessment. In this report we used bespoke analysis, an umbrella review of three meta-analyses and case study analysis to identify trends in 1. Exposure to chemical mixtures in UK marine, estuarine and freshwater ecosystems; 2. The major patterns (i.e. extent of additivity) of aquatic mixture effects; and 3. Evidence of exposure and ecological community responses to chemical mixture exposures in marine and freshwater environments. From these analyses we conclude the following patterns to address questions relevant to mixture impact assessment.

1. *Current position on exposure to chemical mixtures in aquatic ecosystems in the UK.* We used a publicly available dataset from the Environment Agency Open WIMS Water Quality Archive to identify the major chemicals involved in toxic mixtures based on chemical monitoring in water in England. This dataset contains measurements mainly for chemicals measured under the Water Framework Directive for 27,158 samples, of which 87% are for rivers (n=23,592), 11% for marine (n=2,300) and 3% for estuaries (n=679). Using a threshold of significant contribution to a mixture of 1/10 of the HC<sub>50</sub> derived from a species sensitivity distribution for that chemical, 2% of samples have more than two chemicals that have a chemical concentration above the effect threshold indicating the presence of a potentially “toxic” mixtures. Overwhelmingly these “toxic” mixtures were for river samples (97%), with limited toxic mixtures for marine (~3%) and estuarine (<1%). Lowering the threshold to 100th of the HC<sub>50</sub> indicates there are 36% of samples that have more than one chemical that exceed this threshold. The greater percentage of “toxic” freshwater samples compared to the overall percentage, suggests greater chemical pollution pressure for rivers compared to marine and estuarine ecosystems. The most common measured chemicals indicated as contributing to the “toxic” mixtures are metals like Zn, Cu, Al, and Pb, and some specific PAHs. However, these are the chemicals most commonly measured in regulatory monitoring. Other emerging substances groups such as PFAS, pharmaceuticals, plasticisers, some newer pesticides and microplastics are far less commonly quantified and in some case lack the required SSD models needed to be included in the assessment. In mixtures that have more than one chemical exceeding the specific threshold, the most toxic chemical always contributes  $\geq 24\%$  of the total “toxic pressure”.
2. *Percentage of mixture studies showing additive, synergistic and antagonistic effects.* We conducted an umbrella review to summarise the findings of three meta-analyses conducted for three different publication periods. In each meta-analysis, effects measured in published mixture toxicity experiments were compared to the expectation of additivity for the tested mixture, with the aim of identifying the frequency and magnitude of synergism and antagonism in published studies. Despite



different publication periods, chemical groups, study domains, and search strategies, the three meta-analyses (by Beldon et al. 2007, Martin et al. 2021, and Cedergreen et al. 2014) found common trends in mixture effect relationships with additivity. Each found that the number of cases of synergism and antagonism when assessed against additive models was lower than reported by the primary paper authors. This finding indicates that many study authors misinterpret additive effects as synergism. When robustly reanalysed in the three meta-analyses, between 10-25% of experiments showed significant synergism or antagonism. Interaction patterns varied by chemical, species, endpoint, and conditions. Metal-metal mixtures were often antagonistic, while chemicals affecting xenobiotic metabolism, like organophosphates and azole fungicides, were linked to synergy.

3. *Evidence for the magnitude of predicted versus measured mixture effects in the field.* Based on a review of the literature, various modelling and measurement approaches identified plausible ecological risks from chemical pollutants in field studies. Increased toxic pressure from chemical mixtures often led to changes in community structures of some taxa. Most assessed case studies indicated only a weak relationship between mixture complexity and predicted risk to biodiversity – as would be expected, as there are many other pressures that can affect aquatic communities which make it difficult to detect chemical effect signatures among the range of other effects seen. The presence of multiple pressures makes cause and effect linkages for ecological changes a major challenge. Large-scale analysis and the use of diagnostic techniques using appropriately selected biomarkers and biosensors both show promise as tools to establish this mechanistic causality.

Overall chemical mixture research in marine and freshwater ecosystems is an established field. This maturity provides a strong base from which to identify the scale of the aquatic mixture issue and approaches for its management. Many of the relevant principles are common to different environments. Thus, while nuanced differences exist between marine and freshwater, such as how chemical conditions influence bioavailability and the types of species exposed, many key principles are common. For example, as found in Task 1, the nature of the chemical present in marine, estuarine and freshwater habitats are often similar, although more often locally at higher concentrations in freshwaters. Patterns of marine and freshwater species chemical sensitivities have also been shown by meta-analysis to be broadly similar, although systematic, case specific differences may be possible for some chemicals. Additive mixture effects have been shown to predominate at similar frequency in both habitats. When mixtures show synergistic or antagonistic effects, then similar chemicals are often involved. Evidence linking predicted mixture effects to ecological impacts are better established in freshwater versus marine ecosystems, although this is mainly because river habitats are more often studied. What marine evidence exists points to similar linkages.

From the reviews conducted, we can identify areas for progress in chemical mixture research and management. These areas include simple steps such as open data sharing; using mechanistic toxicology to identify chemicals that may cause synergistic/antagonistic effects by changing toxicokinetics and toxicodynamics; and longer-term ambitions, such as increasing the scope of chemical monitoring to a greater range of chemicals; further increasing the number of species sensitivity distribution availability and using genomic data to identify cross species vulnerability to mixture effects.



## Glossary

**CA** – Concentration addition - A additive mixture model that assumes chemicals share the same mode of action

**CIP** – Chemical Investigation Program – A river and effluent monitoring program for freshwater funded by UK Water Industry Research

**CEFAS** - Centre for Environment, Fisheries and Aquaculture Science

**EA WIMS** - Environment Agency Water Quality Archive – A data holding of Environment Agency aquatic chemistry data

**CYP45** - Cytochrome P450 – Enzyme involved in phase 1 xenobiotic metabolism

**EA** – Environment Agency

**ECHA** – European Chemicals Agency

**GC-MS** – Gas Chromatography Mass Spectrometer

**HC<sub>5</sub>** - Hazardous Concentrations for 5% of species derived from a species sensitivity distribution

**HC<sub>50</sub>** - Hazardous Concentrations for 50% of species derived from a species sensitivity distribution

**HQ** – Hazard Quotient derived as the ratio of exposure and hazard for risk assessment

**HQ<sub>max</sub>** - Maximum Hazard Quotient for any chemical in a mixture

**HQ<sub>mix</sub>** – Sum of the Hazard Quotients for all chemical in a mixture

**IA** – Independent Action

**IPQ** - Index of Prediction Quality - A ratio of actual versus predicted mixture effect

**LC-MS** - Liquid Chromatography Mass Spectrometer

**MDR** - Model Deviation Ratio - A ratio of actual versus predicted mixture effect

**PAH** – Polycyclic Aromatic Hydrocarbon

**PBDE** - Polybrominated diphenyl ethers

**PCB** - Polychlorinated biphenyl

**PFAS** - Per- and polyfluoroalkyl substances

**POP** – Persistent organic pollutants

**REACH** - Registration, Evaluation, Authorisation and Restriction of Chemicals – The main regulation for the regulatory management of chemicals in Europe (including the UK)

**SI** - Simple interaction – A mixture model assuming that one chemical that has no effect potentiates the toxicity of another

**SSD** – Species sensitivity distribution – A statistical model that describes the relationship in sensitivities of different species to a chemical

**UKWIR** - UK Water Industry Research

**US EPA ECOTOX** - Ecotox database hosted by the US Environmental Protection Agency



## 1. Chemical mixtures in aquatic environments

The chemical quality of lakes, rivers, estuaries and the sea is a topic of high public, media and political interest. Recent political debate and public campaigns have seen the state of the UK water environment feature as a topic of high concern. Chemical pollution of inland and coastal waters results from multiple source inputs including point source emissions from industry and domestic water treatment and diffuse releases from agriculture, urban run-off and aerial deposition. Landmark policies and programs to reduce acid rain, restrict certain persistent organic pollutant (POPs), biocides and pesticides and upgrade wastewater treatment plants, coupled to economic shifts in how and where a post-industrial country such as the UK generates power and obtains raw materials and processed products, have all led to change in the nature of water pollution. However, despite these shifts, routes for the entry of familiar and emerging pollutants to marine, estuarine and freshwater ecosystems remain.

Producing a picture of water pollution has been made possible by advances that have increased the range of chemicals that can be measured and lower detection limits of modern analytical measurement runs. Use of such sensitive broad spectrum targeted and untargeted analyses has generated significant data on catchment and regional pollutant concentrations in surface waters (e.g. Altenburger et al., 2019; Hermes et al., 2018; Houtman et al., 2019; Inostroza et al., 2023; Park et al., 2018; Peng et al., 2018; Wilkinson et al., 2022). The available data provide opportunities to understand the nature of the chemical mixtures commonly found in different ecosystems. In the UK, large-scale chemical analysis datasets are available from publicly available sources, e.g. The Environment Agency (EA) semi-and fully quantitative monitoring and compliance data available through the EA Water Information Management System (WIMS) (EA WIMS); the UKWIR Chemical Investigation Programme (UKWIR CIP) measurement data for river waters upstream and downstream of waste water treatment plant (WWTP) outflows for England and Wales and chemical monitoring data from Natural Resources Wales, the Northern Ireland Department of Agriculture, Environment and Rural Affairs, and Scotland from the Scottish Environment Protection Agency. Similar monitoring networks and data exist in other countries such as France (NAIADES), Portugal (SNIRH), The Netherlands (Rijkswaterstaat) and for certain river catchments, e.g. River Elbe.

Because of the need for compliance monitoring within the Water Framework Directive, most of the above datasets are for freshwater (although EA-WIMS does include some estuarine and marine data). That does not mean though that estuarine and marine ecosystems are not monitored. For example, the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) runs marine monitoring programs collecting water quality measurements from transitional and coastal waters from passive sampling devices and spot sampling. However, at present this data is not easily accessible, although it could be requested from the CEFAS MONITOOL project (<https://www.monitoolproject.eu/>) for further assessment. In addition, the British Oceanographic data centre has a MERMAN national database that collates marine water quality data under the Clean Safe Seas Environment Monitoring Programme, providing chemical measurements for pesticides, metals and organic contaminants. This data is available via the European Marine Observation Data Network chemistry portal (<https://emodnet.ec.europa.eu/en/chemistry>).

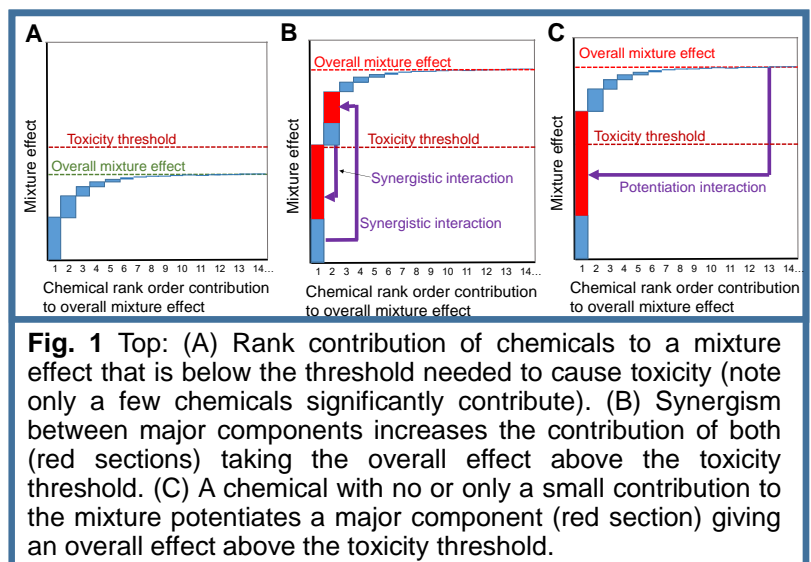


## 2. Mixture effect assessment theory and practice

To interpret chemical measurements in the context of effects, ecotoxicological data for relevant species are needed. Thousands of ecotoxicological papers have been published on chemical mixtures and their effects in marine, estuarine and freshwater environments. Within the mixture ecotoxicology literature, the accepted default assumption for understanding the mixture effects of chemicals is to assume that they work additively without interaction. This assumption is most often taken as the starting point for mixture assessment, providing a default prediction of joint effects that can either be confirmed or refuted in those case where interactions leading to synergism (greater than additive toxicity) or antagonism (less than additive toxicity) occur. There are two major models that are used to make additivity assessments. Mechanism of action is used as the proposed basis for model selection, with Concentration Addition (CA) used for chemicals that act on the same biological target (similar mode of action), and Independent Action (IA) for combinations of chemicals that act independently of each other (dissimilar mode of action) (Van Gestel et al., 2010).

Both the concentration addition and independent action models were initially developed in pharmacology, where knowledge of mode of action is often (although not always) known. In ecotoxicology species, differences in physiology often mean that knowledge of modes of action is uncertain. For example, for chemicals that target a specific receptor, that route to effects will only be relevant if that receptor is present in the tested species and that the receptor plays the role linked to its anticipated function. Whether this is the case is often not known, although methods to approach this challenge are evolving (Spurgeon et al., 2020). This uncertainty means that although mode of action can theoretically be considered in selecting an additive mixture model to use in many ecotoxicological cases, often this choice is not mechanistically well-founded. As such a mixture model use in ecotoxicology is best viewed according to the model's mathematical construction, as combined potencies for CA and event probabilities for IA (Jonker et al., 2005; Van Gestel et al., 2010). Considering the use of mixture models in this way, means that use of either model, while theoretically valid, provides only a statistical prediction of effect.

Use of the two models together can be used to provide a mixture prediction effect window (i.e. that the real effect is somewhere in the space between the predictions of the two models). However, because CA generally produces a more conservative prediction of mixture effects, this model is more generally used in risk assessment.



Classic laboratory studies using carefully selected chemicals, where the mode of action is known, have demonstrated the predictive capacity of CA for similarly acting chemicals (Altenburger et al., 2000; Deneer

et al., 1988a; Deneer et al., 1988b; Faust et al., 2001; Hermens et al., 1985) and IA for dissimilarly acting chemicals (Backhaus et al., 2000; Deneer et al., 1988a; Faust et al., 2003). However, as mentioned above, not all mixture studies show additive effects. Using statistical tools such as isobole plots (Cedergreen et al., 2007), nested MixTox models (Cedergreen et al., 2012; Jonker et al., 2005) and toxicokinetic-toxicodynamic models (Bart et al., 2021; Bart et al., 2022) interactive effects including synergism, antagonism, concentration ratio dependence (synergism or antagonism depending on chemical ratios) and effect level dependence (synergism or antagonism depending on predicted level of the effect) can be identified. From an ecosystem protection perspective, synergism represents the most important pattern, as this interaction has the potential to move cumulative effects from below to above an effect threshold (Figure 1 a-c). Cases of synergism may occur both between major components (Figure 1b), or when a chemical that has only a small effect in the mixture increases the toxicity of one (or more) major components in a specific case that can also be known as “potentiation” (Figure 1c).

### **3. Predicting and quantifying mixture effects in aquatic ecosystems**

Mixture effects assessment is not a routine part of current water quality assessment, with chemical risk viewed on a substance-by-substance basis. Monitoring under current policies, e.g. UK Water Framework Directive or marine conventions and programs, does in some cases also include the status of ecological communities. In cases where exposure to chemicals, including as mixtures, are sufficient to cause biological effects, these will be detected as a shift in the observed versus expected community. However, given the range of potential hydrological, climate, chemical and biological drivers of aquatic community effects, moving observation beyond correlation to mechanistic causation is often a challenge.

Despite the challenges of apportionment, there is evidence that chemical mixtures can exert significant impacts on biological communities. At local scale, there are many examples where point source emissions of mixtures of chemicals (i.e. from end of pipe or a specific site) to surface water are linked to effects on the structure and/or function of aquatic communities. The legacy of mining leading to acid mine drainage is linked to impacts on connected river, estuarine and marine ecosystems (Byrne et al., 2013; Rainbow et al., 2011; Rico-Sánchez et al., 2022; Stockdale et al., 2014a; Stockdale et al., 2014b). Industrial emissions from heavy manufacturing can also impact aquatic communities (Amisah and Cowx, 2000; De Jonge et al., 2014), as can localised releases of toxic pesticides from agricultural or other sources (Beynon, 2012; Thompson et al., 2016). Chemicals present in planned effluent releases or combined sewer overflows have also been shown to affect freshwater estuarine and marine ecosystems (Haase et al., 2023; Wear et al., 2021) .

Evidence also exists on the potential effects that non-point source derived (i.e. distributed releases from agricultural or widespread chemical uses) chemical mixtures can have on aquatic ecosystems. In some cases, specific effects are known, such the population level effect of organotins on some marine molluscs (Matthiessen and Law, 2002). However, when exposure is to multiple chemicals, often at low levels, resolving the debate on the role of chemical effects becomes more challenging. Despite the complexity, there are some indications on the extent of effects that a significant proportion of aquatic species, predicted at 20% (EEA, 2020) and 26%, may be at risk due to the potential effect of chemical mixtures



(Posthuma et al., 2018). This risk was supported by the study of Posthuma et al. (2020) who linked the “toxic pressure” of exposure to 24 household, industry, and agricultural chemicals to changes in aquatic community status. These impacts are reflected in national-scale assessments of river biodiversity. Thus while there are positive time-trends of improvement, aquatic biodiversity is still often low in more human influenced (agricultural, urban) areas where pollution inputs (and also other stressor like stream modification and riparian land-use change) are greatest (Qu et al., 2023; Vaughan and Ormerod, 2012).

#### **4. Knowledge and data relevance for marine, estuarine and freshwater ecosystems**

Across the research literature, thousands of papers have been published detailing the presence of chemical mixtures and their predicted or measured effects on aquatic communities. The availability of such datasets and information varies across habitats, depending on the scope, extent, and openness of monitoring datasets, range of species regularly used for toxicity testing, and the sectoral focus on assessing pollution impacts on ecosystem structure and function. Given the physical, chemical, and biological differences between marine, estuarine, and freshwater ecosystems, it is important to consider whether the information and insights derived from datasets for one habitat, e.g. freshwater are applicable to another, e.g. marine. Chemical sources to marine, estuarine, and freshwater habitats differ in their nature and location. However, the continuum of rivers, estuaries, and coastal marine waters can be viewed as an interconnected system, linking inland chemical sources to marine exposures. Chemical mixtures may potentially be more likely to occur at higher concentrations in freshwater ecosystems given the lower dilution capacity in rivers and lakes compared to open marine systems (Keller et al., 2014). Specific conditions in estuaries, slowing of water flow, mixing of freshwater and saltwater (causing flocculation), tidal action, and stratification, can lead to sediment and co-transported chemical depositions that may make estuaries relative hotspots for the presence of persistent pollutants. Moreover, in any aquatic environment, specific sources can result in high concentrations of chemical mixtures being locally present.

Aquatic ecotoxicity testing in both academic and regulatory ecotoxicological studies most commonly uses freshwater compared to estuarine or marine species. Those freshwater taxa most used, namely crustaceans, fish and algae are, however, also found in marine and estuarine habitats, although the species present naturally differ. Conclusions on the patterns of mixture effects drawn from studies conducted with freshwater species (i.e. the majority of the published ecotoxicology literature) would not be relevant for estuarine or marine environments if systematic differences existed in the sensitivity of freshwater versus marine species. Fortunately, there are enough substances for which the sensitivity of multiple marine and freshwater species has been tested for the same chemical to test whether this is the case. Those meta-analyses conducted to assess these relative sensitivities to date have all concluded there is either broadly similar sensitivity (Kyriakopoulou et al., 2009; Yanagihara et al., 2022) or in one case evidence for marginally lower average sensitivity of marine versus freshwater species (Wheeler et al., 2002). Thus, while individual species-specific differences in sensitivities may occur, read-across of the ecotoxicological effects of chemicals and mixtures between freshwater and marine species seems a reasonable approach.

Ecological assessments of mixtures in marine, estuarine and freshwater ecosystems can adopt similar approaches based on chemical measurements combined with mixture



modelling and community impact assessments. The identification of mixture effects in ecosystems can be affected by habitat vulnerability to other stressors. This can make it difficult to link chemical mixture exposures to impacts against the background of other stressor-induced changes, especially as the general rules on the relationships between species adaptation and multiple stressor effects are challenging to resolve (Orr et al., 2022). One way to approach the linkage challenge in field ecotoxicology is to adopt a mechanistic approach. The adverse outcome pathway (AOP) concept, which highlights the link between chemical exposure and physiological and ultimately population effects of a chemical toxicant, can be helpful in this regard. Thus, the use of robust biomarkers with a clear mechanistic link to observed effects can provide diagnostic evidence on the role of chemical exposure in an observed adverse effect. For mixtures, biomarker evidence can be a challenge as the mixture may act through more than one AOP. This is where well-chosen batteries of biomarkers with known links to multiple major toxicokinetic and toxicodynamic pathways can be valuable. The current marine ecotoxicology literature already gives examples of such use that are highlighted in our response to the research questions we address (notably Question 3).

### **Overall approach to assessing mixture effects in surface water environments**

Consistent with the Invitation to tender, we have undertaken a systematic assessment of the literature to assess the current state of knowledge on the range of chemical mixtures present in waters, their potential patterns of joint effect and the consequences for ecosystem quality. Previous meta-analyses and our own literature searches indicate there is significant open literature and data (100s to 1000s of relevant papers and large-scale datasets). Because of the magnitude of information and the complexity of the brief, which asked for consideration of the mixture effects in rivers, estuaries and coastal areas, at different levels of biological organisation (e.g., ecosystem to genomic) and in combination with other stressors, we have focussed on clearly tractable questions related to the topics, making use when possible, of existing meta-analyses and consolidated datasets to address the three questions below.

1. What is the magnitude of predicted chemical mixtures in aquatic environments in the UK and what chemicals contribute?

*Approach:* Use of UK chemical monitoring data from EA WIMS that covers measurements for England from mainly freshwater, but also marine and estuarine samples, and an expanded set of recently published species sensitivity distributions, to predict the magnitude of mixture effect and the main chemicals involved.

2. What mixtures have been studied; how frequently do tests show additivity and non-additivity; which chemicals are most common in synergistic cases?

*Approach:* Summarise multiple existing meta-analyses conducted by different authors that cover different time periods, chemical classes and used different study identification approaches to identify consistent trends in mixture effect assessment including the range of chemicals used, species tested and endpoints measured (e.g. effects of different apical endpoints such as survival, growth, different aspects of reproductive success) and prevalence of synergistic/antagonistic mixture effects and chemicals involved in those interactive mixture.



3. What is the evidence for the magnitude of predicted versus measured mixture effects in the field and which chemical and other stressors contribute?

*Approach:* Case studies of published field studies that have modelled and measured chemical mixture exposure and/or ecological effects.

### **Question 1. What is the magnitude of predicted chemical mixtures in aquatic environments in the UK and what chemicals contribute?**

#### *Methodology*

To address Question 1, we have used chemical monitoring data from the Environment Agency Water Information Management System (EA WIMS) for England with effect threshold information taken from species sensitivity distributions (SSDs) for 1,000s of chemical published by Posthuma et al. (2019). Combining this exposure and hazard information allowed us to estimate the magnitude of predicted mixture effect and identify which of the measured chemicals contribute significant risk to the mixture.

The rationale for choosing to work with EA WIMS data as exposure information was that this data is openly available and already well curated to allow timely analysis. EA WIMS includes chemical measurement data for marine, estuarine and freshwater habitats. At present, much of the data is for freshwaters. This focus is primarily driven by the demands of implementing policies like the Water Framework Directive, which require chemical data to be collected from freshwater monitoring locations. Because of the dominance of freshwater data in EA WIMS, any conclusions from our analysis will have greater relevance for freshwater than the less commonly reported estuarine and marine ecosystems. To assess whether trends seen in EA WIMS are repeated in estuarine and the sea, further analysis of water quality datasets for these habitats would be needed. Pollutants in marine habitats are measured in the UK by organisations such as CEFAS and National Oceanography Centre. However, currently data from these marine monitoring programs is only accessible by request via data portals (<https://www.monitoolproject.eu/> and <https://emodnet.ec.europa.eu/en/chemistry>) making them much more time consuming for use in mixture exposure analysis (and beyond the resources available for this project).

SSD modelling is a widely applied tool in ecological risk assessment approaches. SSD are statistical models that use species toxicity data to construct a distribution of sensitivities of species to a chemical that can be used to indicate the potential for the exposure of a substance to cause adverse effects on species within a community or ecosystem. SSDs assume that sensitivities to a stressor, i.e. a chemical, varies between species and that this variation can be represented by a statistical distribution. SSDs are comprised of toxicity data (such as EC<sub>50</sub> values) for multiple species fitted in by an, e.g. log-normal, model. The species included are considered representative of the broader ecological community, and their responses are assumed to be independent of each other. As such SSDs reflect the variation in range of potential for effects on species. However, such effects may not scale directly to impacts on ecosystems, due to compensatory or dependent interactions between species. Additionally, the conditions under which the data were obtained are assumed to be consistent



and comparable across species. Hence, SSDs provide a useful, if simplified expression of the comparative potential of species in an ecosystem to respond to a chemical exposure.

The SSDs we used for our analysis were those for >13,000 chemicals assembled by Posthuma et al. (2019). The models used toxicity data for aquatic species (i.e., algae, crustaceans, molluscs and fish) for numerous endpoints (such as mortality, immobility, reproduction, growth and development). This toxicity data came from multiple sources:

- I. US EPA ECOTOX (<https://cfpub.epa.gov/ecotox/>)
- II. Pesticide Property Database (<https://sitem.herts.ac.uk/aeru/ppdb/en/>)
- III. WikiPharma (<https://pharmaenvironment.org/material/welcome-to-the-wikipharma-database/>)
- IV. ECHA REACH registry (<https://echa.europa.eu/de/information-on-chemicals/registered-substances>).

EA WIMS measurements for UK rivers, estuarine and sea water samples were selected for the most recent 5-year time-period (2019-2023) in the database. This data was cleaned to remove other water quality determinants (such as water hardness and pH) and concentration units harmonised for detected chemical measurements (as mg/L). Those chemicals which were present at concentrations below the analytical detection limit have been excluded from the mixture assessment, in alignment with the methodology to assess mixture effects in Spurgeon et al. (2022). This was due to the uncertainty in their measured concentrations in the environment. Exclusion means analytical measurement methods for certain chemical groups could be limiting the substances detected in mixtures above an effect threshold for effects. However, overall, the number of such cases is likely to be small.

**Table 1: Number of water samples and mixture samples (total and above effect threshold) within the EA WIMS dataset.**

Water sample type	Number of samples	Number of mixture samples	Number of mixture samples above effect threshold
River	23592	18138	305
Estuarine	679	460	9
Marine	2887	2300	1
<b>Total</b>	<b>27158</b>	<b>20898</b>	<b>315</b>

Chemicals in EA WIMS include metals/metalloids, polycyclic aromatic hydrocarbons (PAHs), pesticides, persistent organic pollutants (POPs), pharmaceuticals, organic solvents, per- and polyfluoroalkyl substances (PFAS), phthalates and plastic polymers. Major cations and nutrients were excluded from the chemical mixture effect assessment, as these have very high thresholds of ecotoxicological concern and are more often modifiers of the bioavailability of other chemicals, rather than being a toxic stressor in their own right. When bioavailable forms of metals were reported alongside total metals, e.g. water soluble and total metal, the water-soluble concentrations were removed to avoid double counting of substances for a



sample. The resulting list of selected chemicals in the dataset was mainly comprised of pesticides (n=90), POPs (n=57), metals/metalloids (n=21) and PAHs (n=19).

For the toxic pressure assessment measurements above limits of detection were matched with the SSD models for aquatic species for that chemical. We chose to use the acute SSD for the substance, derived from acute  $EC_{50}$  species testing data, as this has the most reported directly usable effect concentrations. This acute SSD was constructed using only data for studies that have generated measured or predicted median concentrations for effects on mortality. From the chemical SSDs, the  $HC_{50}$  hazard values (i.e. the hazardous concentration expected to have an effect on 50% of species) were retrieved for each chemical. When making comparative studies of chemical effects, using the  $HC_{50}$  is preferred because these values are more reliable than values like the  $HC_5$  (hazardous concentration for 5% of species) which are traditionally used as regulatory risk assessment thresholds, as the higher value has lower uncertainty since it is positioned in the centre of the SSD model. Because  $HC_{50}$  values were higher than many measured concentrations, we next used this robust value to generate a lower derived value (10<sup>th</sup> of the  $HC_{50}$ ) as the threshold for identifying significant chemical contribution to a mixture effect.

Exposure data for a total of 272 chemicals were retrieved from the EA WIMS dataset. A total of 27,158 samples were extracted. The samples refer to individual collections across the EA monitoring network and investigation sites, with most being river water (87%, n = 23,592), followed by seawater (11%, n = 2,300) and estuarine (3%, n = 679) (Table 1). Samples may be taken more than once from the same location over the monitoring time-period (2019-2023).

Of the WIMS reported chemicals, 76% (n = 204) had a SSD model available from which the effect threshold as a 10<sup>th</sup> of the  $HC_{50}$  value could be derived (Appendix 1). SSDs were available for the large majority of measured metals, pesticides and pharmaceuticals, with the main gaps for specific congeners among groups of POPs, such as individual PCBs, PBDEs and PFASs. Hazard quotients (HQs) were determined for each reported chemical concentration (exposure) for each of the 204 possible chemicals in WIMS by dividing the exposure concentration by the relevant 10<sup>th</sup> of the  $HC_{50}$  value for that substance. An HQ value  $\geq 1$  indicates that a chemical concentration exceeds the chosen effect threshold, a value  $< 1$  that it does not. The concentration addition model was used to calculate the mixture effect from the individual substance HQ values to derive a  $HQ_{mix}$  value for each sample as the  $\Sigma HQ$  values for all chemical in a given sample. The maximum HQ value ( $HQ_{max}$ ) within each mixture sample was also calculated to assess the contribution of the most toxic chemical (chemical with the highest HQ value) to the mixture effect on aquatic species. Comparison of  $HQ_{max}$  and  $HQ_{mix}$  allowed the toxic pressure of the most important chemical to be assess as a proportion of the overall mixture effect.

### *Findings*

From the 27,158 samples, 77% of these samples were mixtures (n = 20,898) and 23% had only one chemical measurement (Table 1, Figure 2). The frequency of mixture detection was similar for freshwaters (77%) and estuaries (80%), but slightly lower for marine waters (67%) (Table 1). That these different environments showed similar levels of mixture prevalence suggests that all aquatic ecosystems are subject to a broadly similar extent of mixture toxicity



pressure. This is surprising for marine systems given the far greater dilution than for freshwaters in the UK. Marine sites in EA WIMS are, however, often coastal which may explain why mixture exposure are detected as such sites will be closely linked to river discharges. In open seas, the situation may be different as greater dilution has a greater potential to lower chemical concentrations to reduce exposure. This hypothesis, however, needs robust testing using a more comprehensive marine chemical dataset.

A weak significant correlation was observed between sample  $HQ_{mix}$  and the number of chemicals measured in a sample ( $R^2 = 0.0041$ ,  $p$  value  $<0.01$ ) (Figure 3). This positive trend indicated that toxic pressure increased with the number of chemicals in a mixture at low to moderate complexity (2-10 chemicals). As complexity rose further ( $>10$  chemicals), however, this increase was not clearly associated with higher toxic pressure (Figure 3).

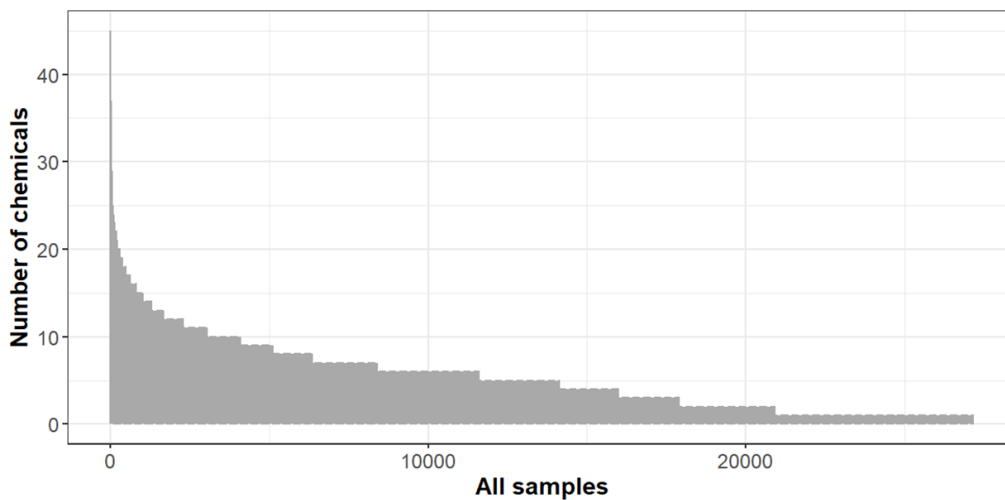


Figure 2: Number of chemical measurements in all samples in EA WIMS.

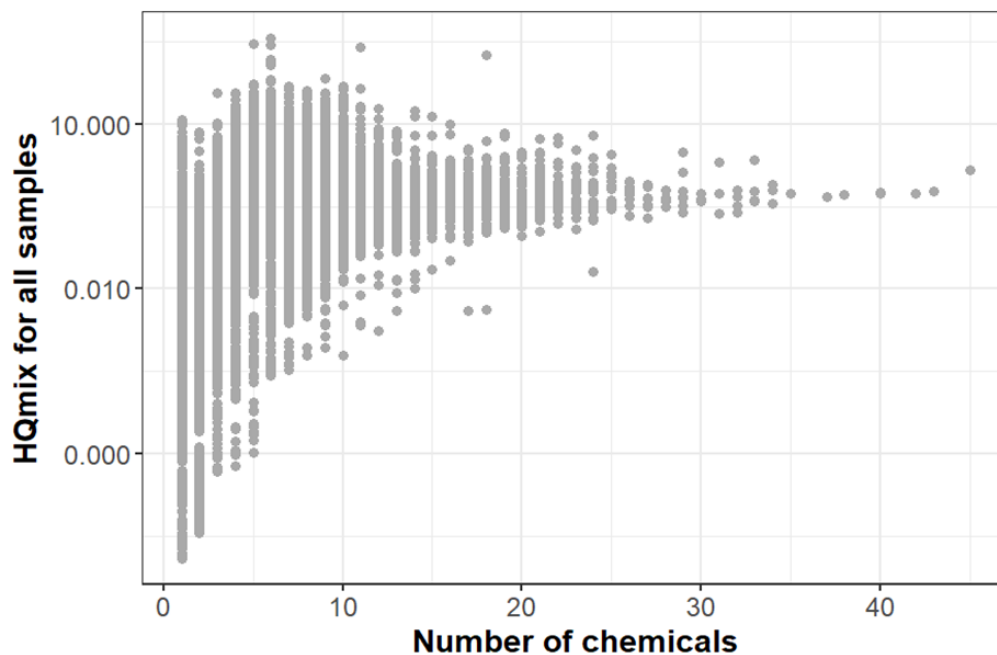


Figure 3: Number of chemicals measured in a sample and the sample  $HQ_{mix}$ .

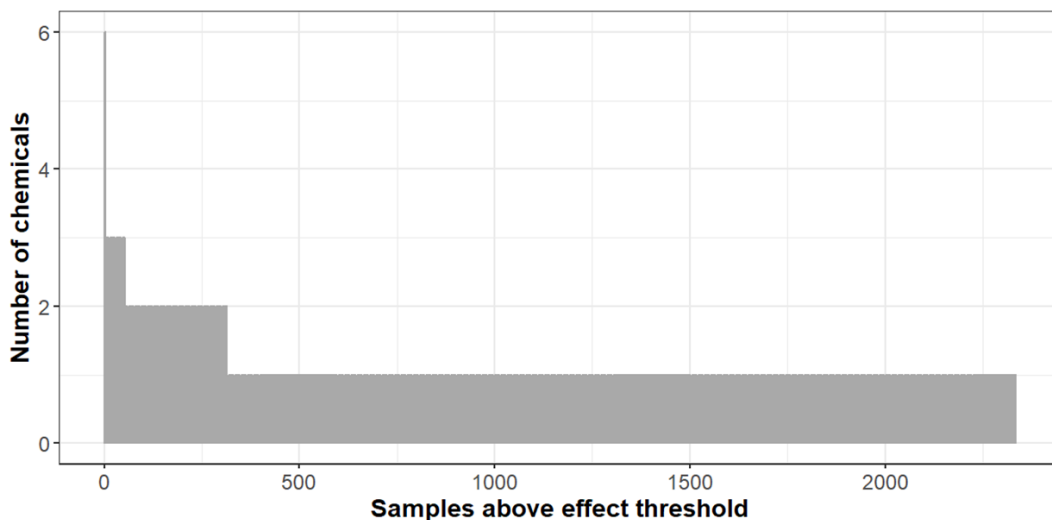


Figure 4: Number of chemical measurements within samples above the effect threshold ( $10^{\text{th}}$  of the  $HC_{50}$  value) in the EA WIMS dataset.

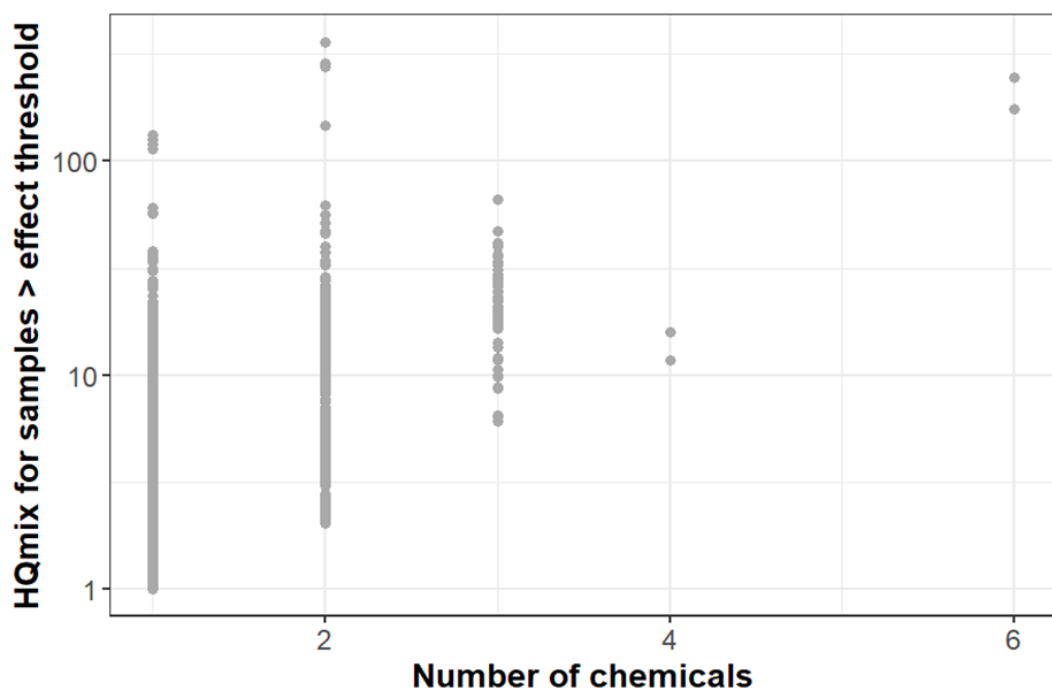


Figure 5: Number of chemicals measured within samples above the effect threshold ( $10^{\text{th}}$  of the  $HC_{50}$  value) in the EA WIMS dataset and the sample  $HQ_{mix}$ .

Of the 2,333 samples with chemical concentrations above effect threshold ( $10^{\text{th}}$  of the  $HC_{50}$  threshold), 86% ( $n = 2,018$ ) had only one chemical measurement above the  $10^{\text{th}}$  of the  $HC_{50}$



threshold), while 14% (n = 315) had 2 or more indicating a potentially toxic mixture exposure (Appendix 1, Figure 4). Most, 97% (n = 305), of these “toxic” mixtures were river water samples, ~3% marine (n = 9) and <1% estuarine (n = 1). This greater dominance of river water samples as toxic mixtures compared to their proportion in the overall database supports the hypothesis that rivers may more often experience high chemical mixture pressure compared to marine ecosystems, however, this assertion would benefit from further assessment using a greater number of marine samples. Comparing the  $HQ_{max}$  values for the most important chemical to the  $HQ_{mix}$  indicated that the most toxic substances contributed between 35-99% of the mixture effect. A weak significant trend ( $R^2 = 0.13$ , p value < 0.01) was observed between sample  $HQ_{mix}$  and number of chemicals in the samples above the effect threshold, indicating that greater complexity increases mixture toxic pressure, although with higher uncertainty (Figure 5).

For samples with chemical measurements above the 10<sup>th</sup> of the  $HC_{50}$  effect threshold, these mixtures most commonly included metals, PAHs and pesticides. Cu and Zn were the substances that are most commonly present in such mixtures (71% of “toxic” mixture samples, n = 225), followed by Al and Zn (22%, n = 69), Al and Cu (12%, n = 37), Pb and Zn (11%, n = 35) and Pb and Cu (4%, n = 14) (Figure 6). In 3% of samples (n = 11) the mixtures above threshold included two PAHs, Benzo(b)Fluoranthene and Benzo(g,h,i)Perylene (Figure 6). Because of the limited number of “toxic” mixture samples for marine and estuary environments (one only in each), it was not possible to state whether the identified mixtures were also the most common in these ecosystems. However, metals and PAHs are recognised as widespread pollutants that have multiple legacy and contemporary sources of input. Hence, it is reasonable to expect them to be widely present in estuarine and marine ecosystems.

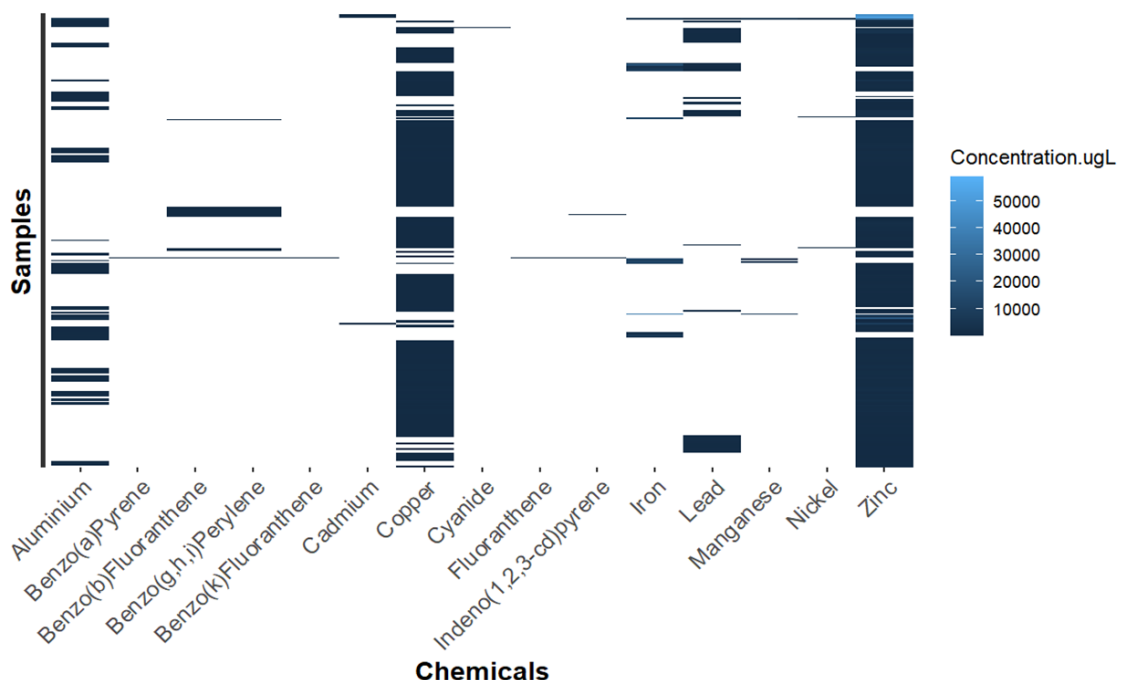


Figure 6: Heat map of chemicals measured within mixture samples in EA WIMS.

Lowering the effect threshold to identify those chemicals making a significant contribution to mixture toxic pressure to 100<sup>th</sup> of the HC<sub>50</sub> value, increased the number of samples with chemicals above this threshold to 13,811. Of these, 46% (n = 6,370) included a contribution above the lower effect thresholds for 2 or more chemical, of which, 96% are river waters (n.b. river waters are 86% of all samples). The most important chemical contributed to 24-99% of the mixture toxic pressure across the 6,370 mixture samples. The chemicals above threshold in the mixture exposure samples included the 15 chemicals also above the 10<sup>th</sup> of the HC<sub>50</sub> effect threshold and also a further 17 substances. Most substances above threshold are metals (97% of mixture samples, n = 13,451), followed by PAHs (6.1%, n = 848), pesticides (0.5%, n = 75) and Industrial chemicals (0.1%, n = 9) (Table 2). Lowering the effect threshold to 100<sup>th</sup> of the of the HC<sub>50</sub> also increased the number of pesticides and POPs in mixture samples. The most commonly occurring pesticides were the herbicides, e.g. 2,3,6-Trichlorobenzoic acid, terbutryn, mecoprop and insecticides including cypermethrin (Table 2).

**Table 2: Chemicals within mixture samples with concentrations above a higher effect threshold of 100<sup>th</sup> of the HC<sub>50</sub> value.**

Chemical	Chemical group	Number of mixture samples
Copper	Metal/metalloids	7626
Zinc	Metal/metalloids	6655
Aluminium	Metal/metalloids	3450
Iron	Metal/metalloids	2837
benzo(ghi)perylene	PAHs	843
Lead	Metal/metalloids	643
Manganese	Metal/metalloids	251
Nickel	Metal/metalloids	207
Arsenic	Metal/metalloids	139
Benzo(b)fluoranthene	PAHs	138
Cadmium	Metal/metalloids	81
indeno(123cd)pyrene	PAHs	43
2,3,6-Trichlorobenzoic acid	Pesticides	37
Cyanide	Pesticides	36
Lithium	Metal/metalloids	28
Benzo(k)fluoranthene	PAHs	27
Benzo(a)pyrene	PAHs	14
Chromium	Metal/metalloids	9
Boron	Metal/metalloids	8
Phenol	Industrial solvent	8
Benzo(a)Anthracene	PAHs	2
2,4-Xylenol	Industrial chemical	2
Fluoranthene	PAHs	1
Terbutryn	Pesticides	1
4-chloro-o-cresol	Industrial chemical	1
4-Chlorophenol	Industrial chemical	1
Mecoprop	Pesticides	1
Dibenz(a,h)anthracene	PAHs	1



Naphthalene	PAHs	1
Cobalt	Metal/metalloids	1
Cypermethrin   Zeta-cypermethrin	Pesticides	1
Mercury	Metal/metalloids	1

### *Implications*

Based on measured concentrations exceedance of the 10<sup>th</sup> of the HC<sub>50</sub>, 2% of the 20,898 of samples in the EA WIMS dataset have chemical concentrations above the effect threshold (HQ<sub>mix</sub> ≥ 1). The most common chemicals at these levels contributing to the potentially “toxic” mixture exposures are metals, notably Zn, Cu, Al and Pb. A small number of samples also have mixtures of PAHs at concentrations above the threshold. Both metals and PAHs are common pollutants that have a range of legacy and contemporary sources linked to current and past industrial activities in sectors such as mining, metal processing, energy production and transport, as well as diffuse used in household and consumer goods. Their common presence at levels above thresholds, especially in freshwaters, is consistent with this widespread source intensity.

Lowering the effect threshold to 100<sup>th</sup> of the HC<sub>50</sub> identified exposure above threshold for 2 or more chemicals in 36% of samples. The aforementioned chemicals, as well as further PAHs and a small number of pesticides and industrial chemicals were identified as the main chemicals that contributed to this toxic pressure in these mixture samples. Among samples where mixtures of chemicals exceeded the threshold, 50% of cases contained a binary mixture, with the remainder of greater complexity. Even in the most complex mixtures, the most toxic chemical contributes ≥ 24% of the total mixture toxicity. This contribution is similar to the findings in Spurgeon et al. (2022) who found that the most toxic chemical contributed >20% of the mixture effects in >99% of cases in EA ground water and surface water organic chemical monitoring datasets.

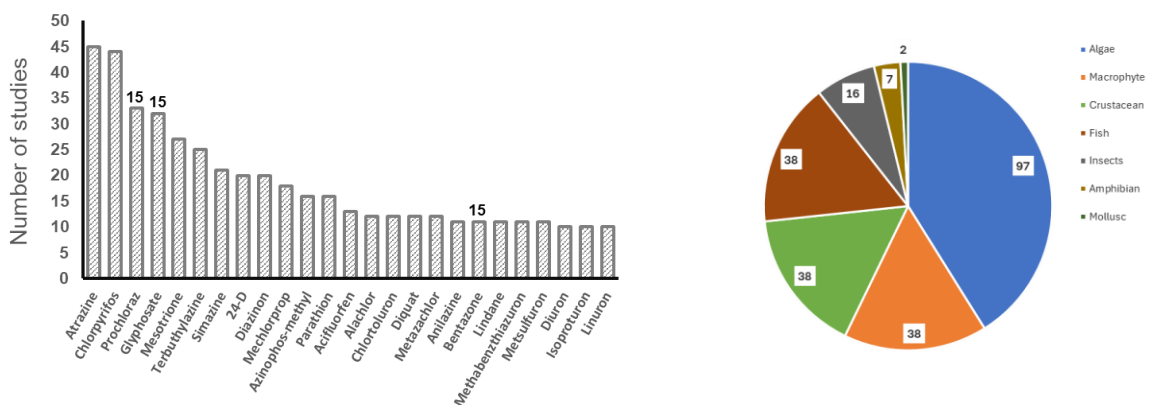
Based on the thresholds (10<sup>th</sup> HC<sub>50</sub>, 100<sup>th</sup> HC<sub>50</sub>), taken from the reported SSD models of Posthuma et al. (2019) these effect thresholds for other chemical groups (pharmaceutical compounds, organic solvents, phthalates, PFAS compounds and plastic polymers) were not observed in mixtures at toxic pressure above the selected thresholds. In some cases, the absence of the chemical could be an artifact of excluding chemical measurements below detection limits. As analytical measurement methods for certain substances improve, the diversity of chemicals included within the mixture effect assessment could increase. Some chemicals were also missed from the assessment as SSDs for these chemicals are not yet available. PFAS compounds other than PFOS and PFOA are notable examples. Other limitations with the EA monitoring program were that not all chemicals are measured, and sampling sites are within England only and largely comprised of freshwater sites. EA monitoring sites are selected based on national agency policy and statutory environmental monitoring requirements for EU directives, as well as reactive monitoring sites for pollution incidents and compliance monitoring for environmental protection regulation. Finally, the surface water environments commonly sampled within the EA monitoring program are predominantly river waters, and thus the inclusion of more marine and estuarine sampling sites within the monitoring network could improve the assessment of mixture effects in these



environments. That said, however, from the data available, it does appear that the mixture of chemicals above effect thresholds were more frequent in river and estuarine samples, compared to the marine waters in WIMS.

**Question 2. What mixtures have been tested; how frequently do tests show responses that deviate from additivity; which chemicals are most common in synergistic cases?**

To address Question 2, we have made use of multiple already published meta-analyses conducted to assess the patterns of joint effect in published ecotoxicological mixture datasets. An extensive literature of mixture ecotoxicological studies are available in the open research literature. The most common type of reported experiments compare mixture effect predictions derived from single chemical data using additive models (CA or IA) to the measured effect of the mixture exposure. Such studies allow the hypothesis of additivity to be tested. If supported, then the observed response will align with the additive prediction. If not, then the actual effect may be less than (antagonistic) or greater than (synergistic) additivity. Multiple meta-analyses have sought to establish the frequency of additivity, antagonism and synergism. Here we review the 3 largest and most recent of these evidence reviews, using their separate outcomes to identify the chemicals and mixture complexities tested, species and endpoints measured, and number and magnitudes of synergisms/antagonisms found. Each study is reviewed in detail and their outcomes tabulated to allow key trends to be identified.



**Figure 7. Left: Frequency of pesticide used in the mixture studies showing all substances used in >10 studies and Right: Frequencies of taxa studies in experiments in the mixture study meta-analysis of Belden et al. (2007).**

*Meta-analysis 1. Belden et al. (2007). How well can we predict the toxicity of pesticide mixtures to aquatic life? Int Environ. Assess. Man. 3, 364-372.*

**Methodology**

Belden et al. (2007) reviewed 303 pesticide aquatic mixture experiments to compare observed effects against predictions for three additive effect models: CA (207 experiments), IA (37 experiments), and a model that the authors termed “simple interaction” (SI) (69 experiments), used to identify case of potentiation (i.e. increasing the toxicity of one chemical



by another itself not having an effect). Studies with 122 different pesticides were included, 69% with herbicides, 7% fungicides and 24% insecticides. Those pesticides used in >10 experiments are shown in Figure 7. These reflect mainly past use compound groups now highly restricted for use, e.g. triazine herbicides (e.g. atrazine, simazine), organophosphate insecticides (e.g. chlorpyrifos, diazinon, parathion), and phenylurea herbicides (chlortoluron, diuron, isoproturon, linuron). Of the 15 most commonly used herbicides, fungicides and insecticides in the UK in 2020, the Belden et al. (2007) dataset includes studies with only 3 of 45 active ingredients, reflecting both the age of the meta-analysis and turnover of pesticides in widespread use since its publication. Species used were from 7 taxonomic groups. Plants (algae and macrophytes) were used in 57% of studies and fish and crustaceans in 17% each, with the remaining groups (molluscs, insects, amphibians) rarely tested.

To assess observed effects against the additive prediction, a Model Deviation Ratio (MDR) was calculated from the expected/observed effects. The threshold used for identifying synergism was a 2-fold change (either up or down) in the potency of the mixture compared to the additive prediction. Thus, an MDR of <0.5 for an experiment (i.e. an observed effect twice the predicted effect) was taken as the threshold for synergism; and an MDR >2 (i.e. observed effect half the predicted effect) as the threshold for antagonism. There is no formal threshold for defining a “significant” deviation of an observed versus and predicted mixture effect. Other meta-analyses have used different thresholds. For example, the review of metal mixtures conducted by Vijver et al. (2011) used a 0.2-fold change as a threshold for identifying interactions, and as a result a higher proportion of non-additive mixtures was identified. However, all three of the large meta-analyses selected for comparison consistently used a 2-fold cut off as the threshold for “significance” making it easily possible to compare the frequencies of synergisms or antagonisms of this scale across studies.

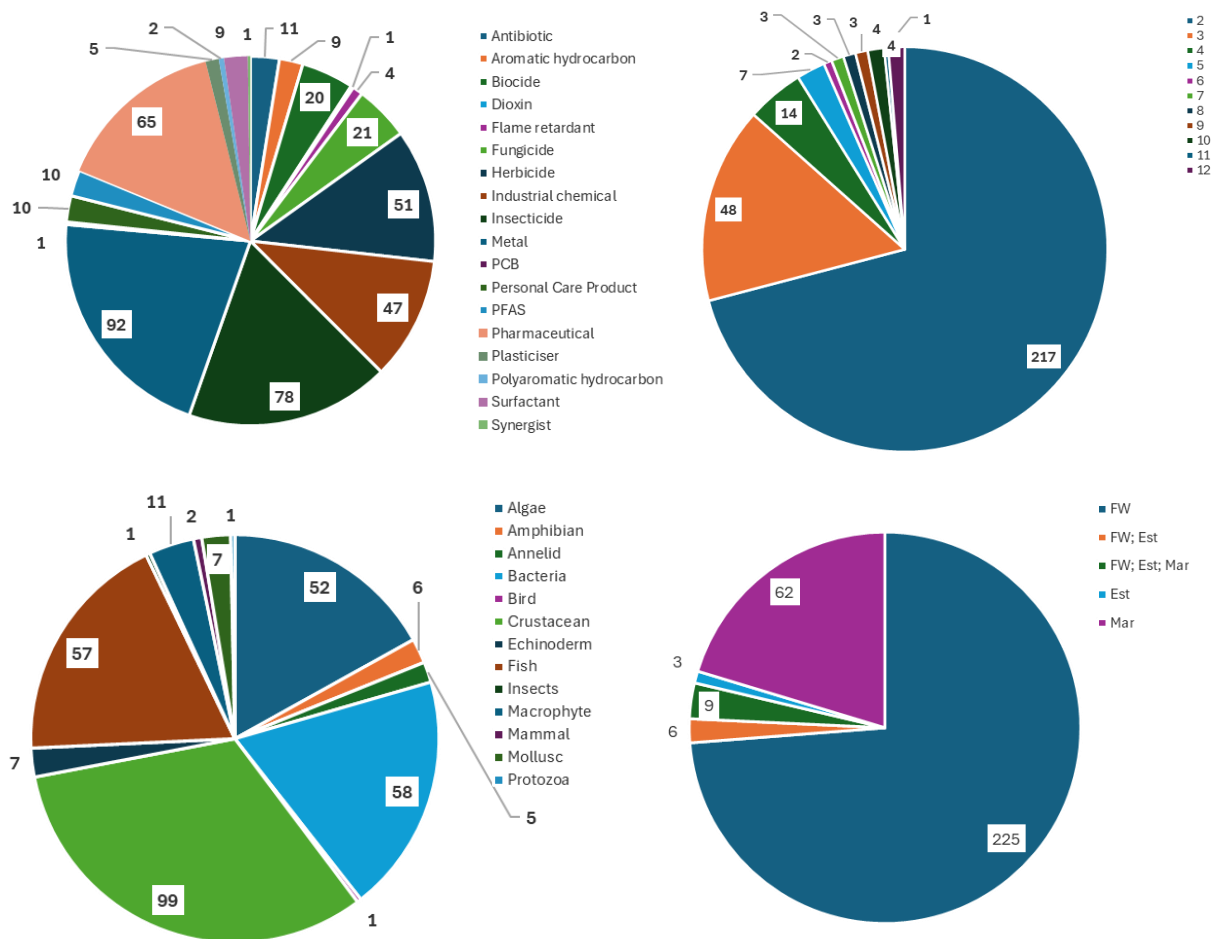
Against the 2-fold cut-off, 88% of the experiments had observed effects within a factor of 2 of additive predictions (MDR 0.5–2.0). Median MDR was ~1 (i.e. there was a balance of studies showing values <1 and >1). Of studies showing >2-fold difference in actual and predicted effect, 6% had MDRs <0.5 indicating synergism and 6% an MDR >2 indicating antagonism, i.e. similar frequencies of both types of interaction. For the potentiation studies using SI as additive model, 80% of experiments had an MDR <2 despite a possible bias towards experiments likely to show synergistic interaction.

### *Interpretation*

The study of Belden et al. (2007) was at the time of publication the largest meta-analysis of observed versus additive predicted mixture effects. The study set benchmarks for identifying “significant” synergism or antagonism based on a 2-fold change in the potency of the observed compared to additive model predicted mixture effects. Overall, the analysis identified an approximate 10% frequency of “significant synergism/antagonism. These two response patterns were approximately equally common indicating an equal probability of both for any theoretical mixture. The study did not, however, provide any clear mechanistic basis for identify the probability of interaction for untested mixtures.



*Meta-analysis 2. Martin et al. (2021). Ten years of research on synergisms and antagonisms in chemical mixtures: A systematic review and quantitative reappraisal of mixture studies. Environment International 146, 106206.*



**Figure 8. Top Left: Chemical classes included in mixtures; Top right: Mixture complexities; Bottom Left: Taxa exposed; Bottom Left: Habitats of tested species for experiments in the mixture study meta-analysis of Martin et al. (2021).**

### Methodology

Martin et al. (2021) followed a similar approach to Beldon et al. (2007). However, the period of literature covered by these authors was completely separate - the 10 years from 2008-2017 versus pre-2007. As such the two studies act as temporal repeats of each other, meaning that if any consistent trends identified are independently supported. Compared to the earlier study, Martin et al. (2021) collated literature mixture study data for a wider range of chemicals and for both mammalian toxicological and ecotoxicological effects (although here we focus on the aquatic ecotoxicology studies only). Papers for assessment were identified by searching for online resources using a set of mixture toxicology terms and the identified literature screened and relevant data extracted. Similar to Beldon et al. (2007), Martin et al. (2021) compared the observed to the additive predicted mixture effects to



generate what they termed the “Index of Prediction Quality” (IPQ) – a metric similar to the MDR. An IPQ indicating a greater or less than 2-fold difference in mixture potency was used as the threshold to identify “significant” synergism or antagonism.

Pesticides were included in one third of all experiments, with insecticides most commonly tested, followed by herbicides and fungicides (n.b. compared to Beldon et al. (2007) there is a trend towards greater testing of insecticides compared to herbicides). Metals were also commonly studied (20% of experiments). Pharmaceuticals and industrial chemicals (including solvents and ionic liquids) were each used in 10% of experiments. Established pollutants less commonly studied included persistent organic pollutants, with few studies on PAHs, PCBs, dioxins, PBDEs, although PFAS were better studied (Figure 8).

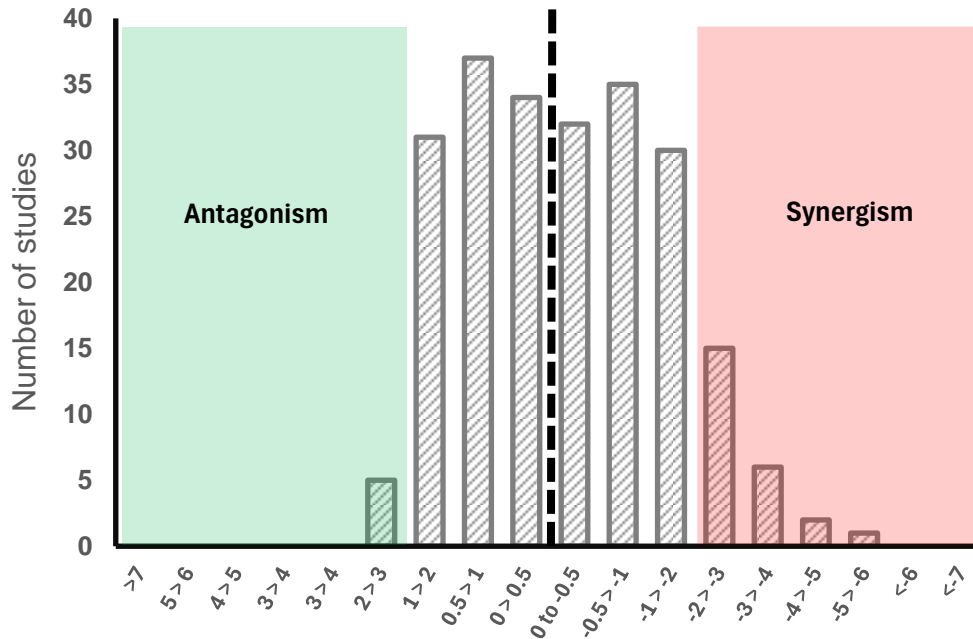
### *Findings*

Binary mixtures were 75% of all tests. Tertiary mixture studies were also quite common, but studies beyond tertiary complexity were rare (Figure 8). Simultaneous exposures were >99% of experiments. Only 0.25% of studies evaluated joint toxicity after sequential exposure. Almost 50% of mixture tests were conducted with either cladocerans or bacteria. The common use of these species can be explained by the availability of standardised methods for testing, e.g. for *Daphnia sp.* and *Ceriodaphnia sp.* and for luminescent bacteria, e.g. *Vibrio fischeri*, and the high throughput capacity of these tests which allows experiments of the scale needed for mixture studies, n.b. a fully factorial binary mixture experiment with a control and 5 exposure concentrations for the two single chemicals and equitoxic mixture with 5 replicates would require 80 experimental units. Fish were the next most common taxa tested, with many studies using the high throughput zebra fish embryo assays and also *in vivo* tests in other species. Smaller numbers of studies were reported for important aquatic taxa such as insects, mollusc amphibians. Studies with groups like fungi and nematodes were absent (Figure 3). Freshwater species (e.g. the cladoceran, insects, algae, amphibians, and the majority of fish, annelids and molluscs) accounted for 75% of studies; marine species were ~20% of experiments, this included those with marine luminescent *Vibrio* bacteria and studies in echinoderms, marine mollusc and fish. Estuarine and Diadromous species were used only in a small number of experiments (Figure 3).

The Martin et al. (2021) study identified data for 1220 experiments from 761 publications. Of these, in 557 (46%) the original publishing study authors self-claimed in the discussion of their results that their study showed deviations from expected additivity, stated through use of words such as synergism, antagonism, “interaction” or “potentiation”. The remaining 663 (54%) experiments were self-reported by the authors as “additive”. Author identification of almost half of all experiments as non-additive, was seen as inconsistent with previous finding of lower levels of >2-fold deviation from additivity. To confirm that all reports of synergism, antagonism or potentiation were fully supported, Martin et al. (2021) reanalysed those author self-reported non-additive studies. Of the 557 author reported interactive studies, 169 could not be reanalysed due to the limited reporting of the data. Of the 388 studies that could be reanalysed, 85 (22%) were for mammalian toxicology and 303 (78%) ecotoxicology, of which 228 were for aquatic species. Following Beldon et al. (2007), the reanalysed author self-reported non-additive experiments were identified as synergistic or antagonistic when an IPQ indicating a >2-fold difference between the observed and predicted effect (>2 for synergism,



>2 for antagonism) was found. A frequency distribution plot of IPQ values indicates that, even for the author selected dataset of non-additive experiments, few studies truly showed a >2 fold difference (Figure 9). Only 24 (10.5%) experiments showed >2 fold synergism and 5 experiments >2 fold antagonism. These percentages for true deviation from additivity are far more consistent with Beldon et al. (2007) than the author self-reported percentages.



**Figure 9. Frequency distribution plots of the number of experiments showing different IPQ values (a measure of observed data similarity to additive model prediction) from the reanalysis of author self-reported interactive mixture studies as conducted by Martin et al. (2021); experiments where the comparison of the observed versus predicted effect results an IPQ = 0 are additive; experiments with IPQ >2 (green shaded region) are above the threshold used to identify “significant” antagonism; experiments with IPQ <2 (red shaded region) are above the threshold used to identify “significant” synergism.**

#### *Interpretation*

Based on author self-reporting the experiments collated in this meta-analysis would have suggested a much higher degree of interactive toxicity than found in previous meta-analyses conducted to identify the frequencies of synergism and antagonism. However, a reanalysis of those author self-reported studies in the meta-analysis by Martin et al. (2021) did not support author self-reporting. When reanalysed, the frequencies of synergism found, approximately ~10%, was consistent with that reported in the fully independent study of Beldon et al. (2007). This similarity supports the view that additivity is by far the most common mixture response and also that true synergism or antagonism that change that may double of half mixture potency can occur in, respectively, about 5% of case for each. The study also highlights the frailties of author self-reporting as a valid and robust indicator of true synergism/antagonism.

*Meta-analysis 3. Cedergreen, N., (2014). Quantifying synergy: A systematic review of mixture toxicity studies within environmental toxicology. PLOS One 9, e96580.*

#### *Methodology*

Cedergreen et al. (2014) conducted a further mixture study meta-analysis for using literature for the period <2012 for three types of substances for aquatic species only: pesticides, metals and antifoulants. This study, thus, included a portion of those studies from pre-2007 collated both by Beldon et al. (2007) and from 2008-2012 collated by Martin et al. (2021) and reanalysed those studies self-reported by authors as non-additivity. Papers were identified using search strings that specifically mentioned both the terms “mixture\*” and “synerg\*”. Similar to the other two meta-analyses, experimental data retrieved from identified papers was used to calculate a model deviation ratio between the CA predicted effect and the observed mixture effect. Studies with >2-fold greater observed than predicted mixture toxicity were identified and synergistic, those with a >2-fold lower toxicity as antagonistic. Because the term “synerg\*” was part of the search string, this study cannot be used to identify the frequency of synergy (or antagonism), because many reported additive studies would be missed. Cedergreen et al. (2014) instead focussed on identifying those chemicals commonly linked to synergy and a discussion of the potential mechanistic causes of these effects.

#### *Findings*

Of the identified experiments, 12%, 14% and 36% had MDRs >2-fold different from CA for pesticides, metals and antifoulants respectively indicating observed effects “significantly” different from predicted additivity. Of these, 58%, 21% and 72% respectively were identified as synergistic, with the remainder antagonistic.

Synergistic or antagonistic effects in mixtures result when interactions occur between chemicals that affect 1) the behaviour in environmental media or at the uptake surface to change environmental availability; 2) the accumulation and distribution of one or both chemicals to change toxicokinetics; 3) the metabolic handling of chemicals to change residence time and internal exposure; 4) how chemicals interact with potential target sites to change toxicodynamics (Spurgeon et al., 2010). Cedergreen et al. (2014) used this framework to attribute synergism and antagonism to potential underlying causes.

Five pesticide mode of action groups were over-represented in the synergistic mixtures: organophosphate and carbamate insecticides (cholinesterase inhibitors), azole fungicides (ergosterol biosynthesis inhibitors), triazine herbicides (photosystem II inhibitors) and pyrethroid insecticides (nerve cells sodium channel modulators) (Figure 10).

Organophosphate and carbamate insecticides both target the acetylcholinesterase enzyme active at the nerve synapse. However, there is widespread evidence that these pesticides also inhibit other esterase enzymes (Sanchez-Hernandez et al., 2018). Non-synaptic esterases are involved especially in Phase I, but also Phase II xenobiotic chemical metabolism (i.e. the chain of reaction that all organisms use to transform and ultimately detoxify and remove xenobiotic chemicals from the body). The effect of organophosphates and carbamates on esterases has the chance to reduce the metabolic transformation of other



pesticides, which would lead to long residence times, higher internal concentrations and, ultimately, to greater toxicity.

For azole fungicide, the mode of action is to inhibit ergosterol biosynthesis by inhibiting lanosterol 14 $\alpha$ -demethylase (also known as Cytochrome P450 51) in fungi. Exposure to azole fungicides has, however, also been shown to also inhibit other Cytochrome P450 enzymes, including those involved in Phase 1 xenobiotic metabolism. Such inhibition of first phase metabolic breakdown could lead to slower pesticide breakdown leading to high body loads and, thus, greater toxicity.

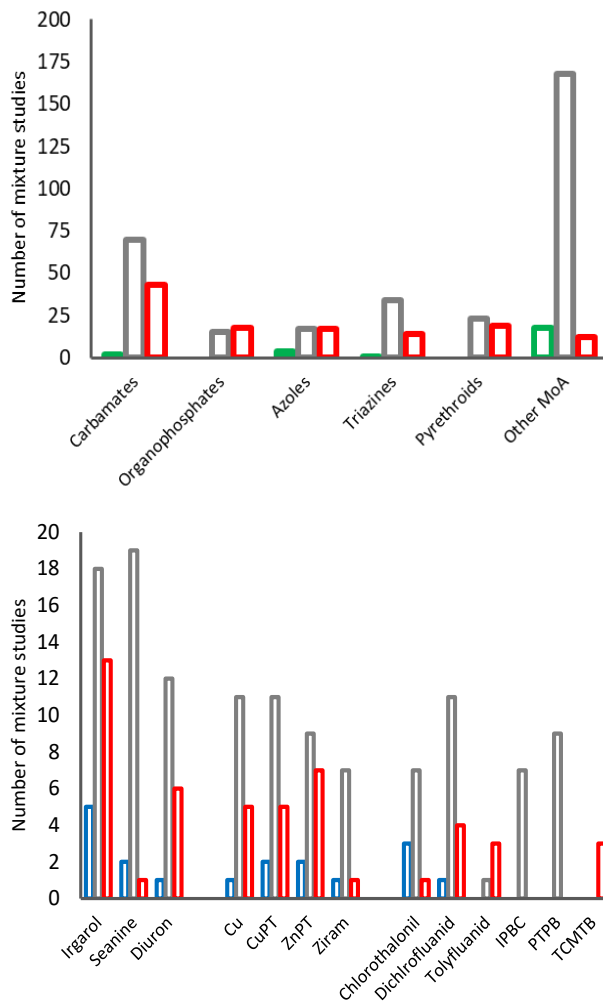


Figure 10. Frequency of synergistic, antagonistic and additive mixture effects for (top) pesticides and (bottom) biocides from the meta-analysis of mixture studies of Cedergreen et al. (2014)

For the other two pesticide classes overrepresented in the synergistic mixtures, there is not such a clear mode of action link to synergy though potential effects on xenobiotic metabolism. Triazine inhibit the photosystem II system in autotrophs. Despite their herbicidal model of action, triazines are also toxic to heterotrophs. The mode of action of these effects is not fully known, but has been linked to oxidative stress and effects of energy metabolism and respiration (Belden and Lydy, 2000). In aquatic species effects on respiration, e.g. ventilation rate, can lead to greater chemical uptake due to increase water volume contact with respiratory surfaces. Any such increase in uptake could again lead to greater toxicity. The mechanism of action of pyrethroid is through interactions with calcium channels in nerve cells. Cases of synergism for pyrethroids were generally when they were exposed with azole fungicides or organophosphates. Pyrethroids are subject to phase I xenobiotic metabolism. Hence, any inhibition of such metabolism may enhance pyrethroid toxicity, without the insecticides themselves being the synergising agent.

There were only four cases of >2-fold synergy for metal mixtures. In each case the metal pair combination was different (Cd+Zn, Cu+Zn, Cu+Cd, Cd+As), with three species (shrimp *Penaeus setiferus*, fish *Gobiocypris rarus*, cladoceran *Daphnia*) impacted. Metal synergism,



thus, seems to show no consistent basis. A greater number of metal mixture studies showed >2-fold antagonism. This is consistent with knowledge about the environmental chemistry and bioavailability of metals, in which competition interactions play an important role in determining metal binding to suspended mineral and organic matter to change bioavailability, toxicokinetics, internal concentrations and toxicity (Niyogi and Wood, 2004).

There were 47 cases of synergy in the antifoulant database. High frequencies of synergy were found for mixtures including irgarol, diuron, Cu, Cu pyrithione or Zn pyrithione, TCMTB, dichlorofluanid or tolyfluanid (Figure 10). Attributing synergy to mechanism of action is more difficult for antifoulants than pesticides, as mechanistic information is not required for active ingredient registration. Interaction leading to non-additivity, mainly antagonism, in antifoulant mixtures containing metals are likely to be similar to those that cause interactions in metal only mixtures. A consistent pattern among many of the other chemicals more commonly showing synergism is the potential to cause energy related metabolic change through oxidative stress. Any such effects may potentially cause effects on ventilation rates that change respiratory exposure. Any mechanistic links between the substance and changes in xenobiotic metabolising enzymes, e.g. phase I cytochrome P450s (for dichlorofluanid and tolyfluanid or Phase II linked glutathione metabolism for chlorothalonil could also provide mechanistic underpinning of synergisms.

	<i>Belden et al. (2007)</i>	<i>Martin et al. (2021)</i>	<i>Cedergreen et al. (2014)</i>
<b>Period of meta-analysis</b>	Pre 2007	2008-2017	Pre 2014
<b>Chemicals covered</b>	Pesticides	All	Pesticides, metal, antifoulants
<b>Toxicology / ecotoxicology</b>	Ecotoxicology	Toxicology and ecotoxicology	Ecotoxicology
<b>Ecosystems covered</b>	Freshwater, estuarine, marine	Freshwater, estuarine, marine, terrestrial	Freshwater, estuarine, marine, terrestrial
<b>Total mixture assessed</b>	303	1,220	194 pesticide, 21 metal, 136 antifoulant
<b>Default mixture model used</b>	Concentration addition, Independent action, simple interaction	Concentration addition	Concentration addition
<b>% interactive mixtures</b>	12%	10.5%	12% pesticide, 14% metal, 36% antifoulant
<b>% synergistic mixtures</b>	6%	6%	7% pesticide, 3% metal, 26% antifoulant

**Table 3: Umbrella summary of the results found in the three meta-analyses of mixture experiments of Belden et al. (2007), Martin et al. (2021), Cedergreen et al. (2014).**



### *Interpretation*

Despite covering different publication periods (<2007, 2008-2017, <2012), different chemical groups (pesticides only; all chemicals; pesticides, metal, antifoulant biocides), study domains (terrestrial and aquatic ecotoxicology; terrestrial and aquatic ecotoxicology and human health; aquatic ecotoxicology) and using different search strategies (all studies; synergy linked studies; author identified non-additivity), the meta-analyses of Beldon et al. (2007), Martin et al. (2021) and Cedergreen et al. (2014) identified some common trends on mixture effect relationships to additivity (Table 3). In all three reviews, authors reported far more cases of synergism and antagonism than are actually found after a robust reanalysis of the data using mainly CA, but also IA and SI, as additive mixture prediction models. This finding indicates that many authors misreport or misinterpret additive effects as showing cases of synergism. Such cases often occur when in studies where authors expose organisms two chemicals as a mixture that would each have an effect at that exposure concentrations used in the study. When the joint effect of this co-exposure is observed to be greater than that for any single chemical alone, this case is regularly erroneously reported by authors as “synergism”, when actually such results can be fully consistent with an additive effect. The reanalyses identified in all cases that <25% (antifoulants in the study of Cedergreen et al. 2014) and in most cases <10% of experiments showed >2-fold synergism or antagonism. Experiments which included a specific chemical in a mixture could each result in different additive, antagonistic or synergistic response patterns, depending on the co-exposed chemical; the species tested; endpoint used and/or environmental conditions of the study. Some classes of chemicals showed a greater likelihood to cause a specific type of interaction. For example, metal-metal mixtures were more likely to be antagonistic, due to competition effects between ions changing environmental bioavailability and/or affecting interactions with uptake sites to alter toxicokinetics. Chemicals known to have modes of action that could be plausibly linked to xenobiotic metabolism are commonly linked to synergy. These include organophosphates and carbamates that can inhibit esterases; azoles fungicides that may inhibit cytochrome P450 enzymes and chloronitriles fungicide, such as chlorthalalonil that may deplete glutathione and so reduce phase II xenobiotic conjugation reactions.



**Question 3. What is the evidence for the magnitude of predicted versus measured mixture effects in the field and which chemical and other stressors contribute?**

To address this question, we conducted an evidence review to identify studies where mixture exposure and/or effect have been assessed in the field. Case studies have been identified in the published literature where field studies have measured the chemical mixture exposure and have sought to link this through prediction and/or *in-situ* effect measurement to ecological impacts. Mixture models predict that as mixture toxic pressure increases the species richness at sites will decrease (Stockdale et al., 2010) (Fig 11). However, both chemical mixtures and other environmental stressors contribute to observed species effects. In the field, such additional factors, including biotic (competition, predation, dietary exposure), physical (waterflow, suspended sediment) and macronutrient variables can all impact organism response to chemical pollution, and these environmental factors are not included within chemical mixture models. Thus, sites with low species diversity/low ecosystem performance will result in species richness impacts at lower mixture toxic pressures than predicted based on just the chemical mixture effect alone (Fig 11).

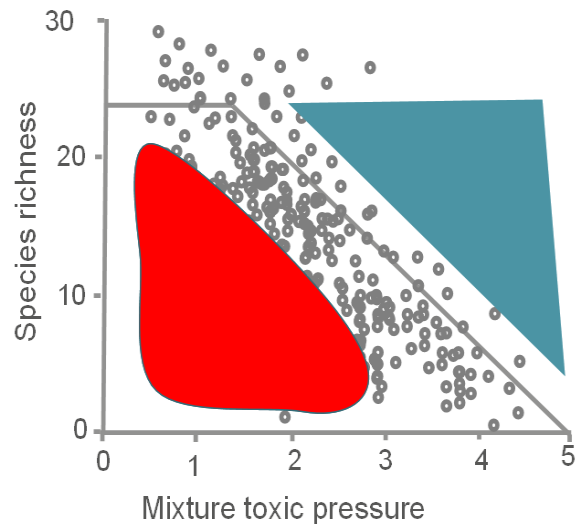


Figure 11. Regression between species richness and mixture toxic pressure (figure adapted from Stockdale et al., 2010). Red triangle shows sites with lower species richness where other environmental factors are impacting ecological response to chemical mixtures.

For a systematic literature review approach, a search string (see footnote<sup>1</sup>) was developed to identify the published literature on studies which compare chemical mixture modelling to field observations on biological effect from chemical exposure. This search string includes terms for chemicals (such as pesticide\*, metal\* and pharm\*), mixture modelling (such as concentration addition and independent action) and biological effects (such as species richness and species abundance).

A total of 372 papers were extracted (date searched: 5/9/24) using the above search term in Web of Science. Out of the 372 papers, only 22% (n=82) of studies were related to marine and/or estuarine environments or organisms. Of these 82 studies, most were related to

<sup>1</sup> Search string for studies looking at relationships between mixture pressure and biodiversity in the field. (TS=(pesticide\*) OR TS=(metal\*) OR TS=(antifoul\*) OR TS=(chemical\*) OR TS=(pharm\*)) AND (TS=(freshwater\*) OR TS=(river\*) OR TS=(marine) OR TS=(estuar\*) OR TS=(aquatic)) AND TS=(mixture\*) AND (TS=(concentration addition) OR TS=(independent action) OR TS=(interaction) OR TS=(synerg\*) OR TS=(antag\*)) AND (TS=(biodiversity) OR TS=(species richness) OR TS=(species diversity) OR TS=(species abundance) OR TS=(species loss) OR TS=(species decline) OR TS=(community decline) OR TS=(community abundance) OR TS=(community diversity) OR TS=(macroinvertebrate\*) OR TS=(biomarker\*) OR TS=(bioindicator\*))



marine environments (71 studies), with fewer estuarine cases (8 studies). Only 2 studies had field observations for both marine and estuarine environments or organisms. Study abstracts and titles of these papers (n= 372) were double screened and from this, 24 studies were identified as having both the relevant exposure and effects data. The criteria to identify the final set of relevant studies included whether studies conducted mixture modelling based on measured chemical concentrations and whether biological effects were assessed for freshwater, marine or estuarine environment site samples. Studies conducted in artificial waters or experimental systems were, thus, not included in the analysis. After full text review 4 of the 24 studies passed the criteria outlined above.

Based on the screening criteria, the selected 4 studies assessed chemical mixture effects in surface waters and linked this to mixture toxicity pressure and biodiversity effects (Bighiu et al., 2020; Ginebreda et al., 2014; Munz et al., 2017; Pereira et al., 2018). All such studies were conducted in freshwaters. There were no studies that assessed chemical mixture modelling and observed biological effects (i.e., species richness and species abundance) to chemical exposure in marine and estuarine environments. Most of the marine and estuarine studies assessed biological responses within experimental systems, where chemical exposures were not comparative to environmentally relevant concentrations and chemical mixture complexity. Furthermore, these studies did not examine population level effects (i.e., species abundance, species loss, species diversity), but concentrated on individual effects. Out of the initial 81 marine/estuarine studies a limited number of studies met some of the required criteria needed to understand mixture effects in the field. One study examined biological effect to chemical exposures above environmentally relevant levels (Echeveste et al., 2016), while another examined biomarker response in relation to chemical exposure in sea water (Echeveste et al., 2016; Lima et al., 2007).

The lack of marine or estuarine studies combining chemical measurements, mixture modelling and biological effect assessment found in our screening, indicates a clear gap in the evidence for predicted vs measured mixture effects in marine environments. This research space does not, however, mean there is not a large body of work investigating pollution impacts in marine and estuarine environments (Lionetto et al., 2021; Mearns et al., 2019). Indeed, there are a number of methods and approaches identified in marine studies that can be used to provide knowledge of some aspects of chemical mixture exposure. Some examples of such papers which were found relevant when removing “modelling” from our screening criteria. Examples of studies identified using these modified search terms are given in case studies 6 and 7 as well as the summaries in Table 4. Limitations due to time and cost meant that a full systematic review of mixture effects with wider screening using broader search terms across multiple literature search databases could not be conducted. For example, removing search terms for mixture modelling from the search string results in a total of 1,305 papers (across all aquatic environments) for screening. Furthermore, grey literature could not be examined which would also have contributed to missing information and resources to gather marine evidence.

### *Findings*

The field survey studies (Case studies 1-4) which were reviewed looked at mixture exposure and aquatic biodiversity impacts. Mixture exposures were taken from field measurements for



multiple chemicals, with pesticides, metals and pharmaceuticals the mostly commonly quantified chemical groups. Biological metrics included endpoints for algae (chlorophyll-a content, photosynthetic capacity, diatom community assemblage, biovolume and taxa abundance) and macroinvertebrates (species diversity, biomass, community assemblages and taxa abundance). Most of the studies applied a concentration addition approach to assess mixture effects (Bighiu et al., 2020; Ginebreda et al., 2014; Munz et al., 2017), except for Pereira et al. (2018) who used an independent action approach. Two different mixture effect assessment approaches have been applied across these case studies, which includes either summing the toxic units (i.e., HQs = exposure/toxicity) of chemicals in a sample ( $\sum$ TUs) or predicting the fraction of species affected by a mixture from chemical SSDs using a multi-substance potentially affected fraction (msPAF) approach.

Across the case studies, there is evidence showing good agreement between predicted mixture effects and observed field biological effects on algae, macroinvertebrates and nematodes (Bighiu et al., 2020; Ginebreda et al., 2014; Munz et al., 2017; Pereira et al., 2018). In some cases, the correlations were observed only for specific taxonomic groups. For example, Bighui et al. (2020) found that only nematode species abundance/richness significantly correlated with predicted mixture effects due to the overriding influence of nutrient enrichment on algae and macrophyte populations. Further, Pereira et al. (2018) found a relationship only between macroinvertebrate taxa abundance and predicted mixture effects, and no clear relationship for algae, which is likely due to the influence of habitat and physical-chemical conditions. This differing results across taxa suggest that in some case chemical mixture effects dominate, while in others, the effect of other stressors on aquatic species can counteract mixture toxicity effects.

The case studies for the relationship between mixture pressure and biodiversity in the field often find that only a few chemicals generally contributed to the mixture effect on aquatic species (Bighiu et al., 2020; Ginebreda et al., 2014; Munz et al., 2017; Pereira et al., 2018). This insight aligns with the findings of Spurgeon et al. (2022) who found that almost always only a few substances contribute to mixture pressure, with the most toxic chemical contributing to  $\geq 20\%$  of the mixture effect in all case. The most toxic chemicals most often found contributing to the mixture effects in the case studies were diclofenac, diazinon and clothianidin (Munz et al., 2017), chlorpyrifos, cypermethrin and cyhalothrin (Pereira et al., 2018) and terbuthylazine, metazachlor, picoxystrobin, mecoprop and pirimicarb (Bighiu et al., 2020).

Most of the identified marine and estuarine studies including chemical mixture modelling and effects were conducted in experimental systems, where chemical mixture complexity and concentrations were different to environmentally relevant levels. In the marine environment there is evidence of biological effects (i.e., oxidative stress and metabolism effects within mussels) via biomarker response to chemical exposure from petrochemical contamination (Lima et al., 2007) (Case study 5). There is also evidence of population level effects (i.e., phytoplankton community effects) from exposure to chemical mixtures of organic pollutants found within sea waters, but at concentrations well above environmentally relevant levels (Echeveste et al., 2016) (Case study 6).



A further set of marine studies were identified that did not match our initial criteria with respect to including both the field chemical measurements as well as the mixture modelling elements that are needed to compare predicted versus ecological mixture effects (Table 4). Within these studies, the most common component missing was the mixture modelling. This gap means that the chemicals measured are related to the quantified biological effects independently, rather than considering their potential joint effects as a mixture toxicity stress. In some cases, quantitative biomarker measurements were used as the measure of biological effect. Depending on the mechanistic foundation of the biomarkers and the chemical modes of actions that they most reliably detect, the use of the measures could be matched against the exposure levels of relevant compounds.

**Table 4:** Marine and estuarine studies that have assessed the effect of chemical pollution exposure to organisms.

Habitat	Study type	Organisms/ Endpoints	Observations	Reference
Marine	Field study	Mussels ( <i>Mytilus edulis</i> ) and Crabs ( <i>Carcinus maenas</i> )	Biomarker response to exposures in the field. Sites with different levels of pollution could be distinguished based on the biomarker response.	(Astley et al., 1999)
Marine	Lab experiment using natural zooplankton communities from the field	Zooplankton – mortality, reproduction and community structure Algae - growth	Zooplankton reproduction and algal growth affected by mixture effects. Levels above environmental relevance found toxic to zooplankton. Observed experimental effects on endpoints were underestimated by 21% compared to predictions from the Independent Action model.	(Jönander et al., 2022)
Marine	Lab experiment with mussels collected from the field	Mussels ( <i>Mytilus galloprovincialis</i> )	Biomarker approach to assess mixture effects. Mixture effects were mostly additive, for effects on energy metabolism impacts and cellular stress.	(Dumas et al., 2024)
Marine	Field study	Shrimp ( <i>Crangon affinis</i> ) – metabolic responses	Biomarker approach to assess mixture effects. Metal pollution impacted metabolic responses in shrimp species in	(Xu et al., 2016)

			organisms exposed in eth field.	
Marine	Lab experiments and field mesocosm	European seabass ( <i>Dicentrarchus labrax</i> ) – growth and survival	Fitness of fish exposed to chemically a mixture of treated crude oil chemicals was impacted (impaired hypoxia tolerance and swimming performance) but growth and survival not impacted.	(Mauduit et al., 2016)
Estuarine	Lab experiments using sediments from the field	Fish ( <i>Solea senegalensis</i> ) – biochemical endpoints	Biomarker approach used to assess mixture effects. Substance specific biomarkers linked to chemical handling mechanisms (metallothionein and cytochrome P450) can be used as indicators of, respectively metallic and organic contaminant exposures.	(Costa et al., 2009)

While there are fewer studies employing mixture modelling approaches in true field compared to experimental studies, a number of methods have been applied widely in aquatic environments to detect the presence of stressors and signs of pollution in both freshwater and marine environments. The PRIMER (Plymouth Routines in Multivariate Ecological Research) software tool (Clarke and Warwick, 2001) has been developed to perform multivariate analyses for assessing changes in marine species community assemblages within polluted areas. Biosensors are also used within marine pollution monitoring, and can monitor biologically derived material (i.e., enzymes, micro-organisms) and quantify chemical pollutants. For example, biosensors have been used to monitor nutrient pollution and the presence of anti-fouling agents, pesticides and endocrine disrupting chemicals in marine environments (Kröger et al., 2002). Monitoring chemical concentrations in marine biota is also widely used to assess marine pollution exposure in sentinel species (Parolini et al., 2023; Poynton et al., 2014; Schäfer et al., 2015).

Biomarkers and omics tools offer further approaches to assess pollution at a sub-organismal level in marine environments (El-Sikaily and Shabaka, 2024; Schuijt et al., 2021). Further, the integration of both can lead to the identification of general and specific indicators of chemical and other stressor modes of action (Nam et al., 2023). For example, Hillyer et al. (2023a) used metabolomics to investigate changes in metabolite in an estuarine invertebrate caused by exposure to heavy metals and PAHs. Linkages of these effects with effects on organism traits, such as body size, were also identified indicating links to population relevant endpoints. At the same sites, multi-omics approaches were also applied to detect both



changes in estuarine bacterial community composition and functional traits as a result of exposure to the legacy metal contamination present (Hillyer et al., 2023b).

Biomarkers can be molecular, cellular or physiological (gene, protein, metabolite expression) indicators that provide information on the impacts of different pollutants and environmental stressors on organism health and used to assess population diversity and structure. Ecotoxicological biomarkers have been applied to signal marine pollution, and their use within marine pollution monitoring has been growing since the 1990's (Depledge, 1994; Handy et al., 2003; Lionetto et al., 2021; Owen et al., 2008). For example, biomarkers have been used to assess PAH pollution and metal toxicity in marine systems in exposed clams (*Ruditapes philippinarum*) (Aouini et al., 2018; Ji et al., 2019; Lomartire et al., 2021). To capture these studies, the term 'biomarker\*' was added to the search string within the literature review. A marine biomarker case study identified is detailed in Case study 6 (Lima et al., 2007). In this work biomarkers were applied to detect key physiological functions that are influenced by both chemical pollutants (PAHs) and other environmental factors (i.e., salinity, temperature and nutrients). Further literature was also identified on the use of biomarkers to study metabolic impacts to marine mussels and shrimp (Xu et al., 2016; Dumas et al., 2024) and biochemical markers indicating the activation of metallothionein and cytochrome P450 systems linked to detoxification pathways in fish (Costa et al., 2009).

Blackwell et al. (2019) (Case study 7) demonstrated the use of biosensors for assessing a biological receptor approach to exposure to chemicals mixture in US surface waters. Here reporter assays were used to detect activity of endpoints, such as binding to known hormone receptors, the activation of which can be linked to adverse apical effects through plausible adverse outcome pathways. Due to the well-defined nature of ligands binding to some of the pathway receptors included in the screening such bioactivation could be used to identify biologically active chemicals in waters. This study detected induction of many receptor mediated pathways in total, but 11 of these accounted for 95% of all cases. Mechanistically linking chemical exposure to activation was only possible for a few chemicals, nearly all with known affinities to the induced receptor, e.g. estrone, 17 $\beta$ -estradiol, bisphenol A, and 4-nonylphenol to the estrogen receptor. Such clear linkage was not possible for most measured compounds (> 700) indicating cosmopolitan activation and a likely effect of multiple chemicals on the assay endpoint (Blackwell et al., 2019). This, while not fully successful in establishing linkages between specific chemicals and their effects, illustrates the potential to apply biosensor approaches within a mixture modelling approach to identify the combined effects of chemicals on known ecotoxicological effect pathways.

### *Implications*

Evidence from the field sample based case studies indicates that prediction from mixture modelling can provide a useful tool to assess the potential for chemical mixtures to impact aquatic ecosystems. This indicates that the use of mixture modelling approach can provide a tractable solution to identifying mixture risks in the field for use in potential risk identification and monitoring. Using different modelling and measurement approaches, the different case studies identified a plausible risk to ecological systems of chemical mixtures. As the potential toxic pressure of the mixture of chemicals present increased, linked changes in the community structures of some, but not an all case all, taxa could be identified. Often the



chemical pressure on aquatic species was indicated to be largely driven by only a few toxic chemicals, as there is only a relatively weak relationship between increasing mixture complexity and the level of predicted risk (i.e. there is a positive relationship, but with extensive scatter between the number of chemicals present and in environmental mixture and the level of predicted risk). Further, the presence of other environmental factors, numerous stressors alongside chemical mixtures that also result in biological effect in aquatic environments make unequivocal linkage a challenge. The exception is when there is a clear mechanistic link between a chemical exposure and an adverse effect. Here use of diagnostic biosensor systems can help close the loop between cause and effect.

### **Progress and the way forward**

When highlighting the risks of water pollution, the non-science media, political figures and public often talk of a “toxic soup” or “chemical cocktail”, indicating a recognition that the chemical challenge to water quality is more than just a one substance issue – as confirmed by our analysis to address Question 1. In the regulatory sphere and even among researchers, aquatic mixture ecotoxicology is often view as a “wicked problem”, too complex to solve. Yet this is not a position consistent with the current state of the science and the available evidence base. Advances in analytical methods mean that it is now possible to identify and quantify the concentrations of 100s or even 1000s of different chemicals in a water sample. While in the past it has been true that we lacked much reliable data on the potential potency of these toxic chemicals, efforts to expand the number of available ecotoxicological benchmarks have begun to fill this gap - with many thousands of SSDs, of varying quality, now available (Posthuma et al., 2019). Mixture model concepts based on additivity have been found to predict actual mixture effects within 2-fold in 80-90% of cases – as confirmed by our analysis to address Question 2. Where these models fail, a multi-stage concept considering bioavailability, toxicokinetics and toxicodynamics can provide the basis for mechanistic attribution of the causes. Within this framework, chemicals that change the environmental bioavailability, bio-uptake or especially xenobiotic metabolism of a second toxic chemical often feature in synergistic mixtures. In the field multiple case studies have linked mixture exposure to adverse effects on aquatic biodiversity, indicating the potential for mixtures acting through different mechanistic pathways to affect ecosystems – as confirmed by the case studies reviewed to address Question 3. Within this knowledge base, are aspects that can underpin and advance knowledge of aquatic chemical mixtures and their effects. Below we summarise the available knowledge and identify opportunities for research innovation and regulatory development in four key thematic areas relevant to the issue: mixture analysis, effect modelling, ecosystem effect prediction and methods for effect attribution.

#### *Advancing the environmental analysis of chemical mixtures in aquatic ecosystems*

The detection of the range of chemicals present in marine and other aquatic ecosystems in time and space is key to characterising the complexity and diversity of the chemical mixtures. Traditional targeted chemical analysis continues to improve the number of substances that can be quantified, the sample throughput and detection limits achieved. This supports mixture identification with specific considerations.



1. Identifying the chemicals that make a substantive contribution to a mixture is defined by both the analyses conducted (i.e. what can be measured) and the data available on effect threshold from which to scale the measured concentrations to calculate toxic pressure. In the 2010s advances in mass spectrometry significantly enhanced the measurement of environmental pollutants in marine environments. These instruments have improved sensitivity and resolution, enabling the detection of trace levels of contaminants previously undetectable. The development and combination of both targeted and untargeted analysis techniques has further advanced environmental studies. Targeted analysis allows for the precise quantification of known pollutants, while untargeted analysis enables the discovery of new and emerging contaminants, providing a more holistic understanding of marine pollution and the mixtures present. With these methods in hand, a more complete view of the pollutants present in aquatic environment is possible.
2. Studies to understand exposure and impacts for chemical mixtures have been conducted in marine, estuarine and freshwater river and lake environments. The majority of these studies are for freshwaters which are routinely monitored for their chemical conditions and biodiversity by regulatory agencies to meet a range of statutory reporting aims. This combination of data is particularly well suited for use in modelling studies of mixture exposure and effects. Commonality of relevant sources, the connectivity of freshwater, estuarine and marine ecosystems and similarities in the taxa, although not species present, means that many of the principles on mixture exposure and effect developed using the richer data available of freshwaters likely hold also for marine ecosystems, e.g. chemical mixtures commonly found; profile of proportional chemical contributions to mixture pressure; overall range of sensitivity of species; prevalence of additive and interactive effects. Environmental monitoring for chemical conditions is also conducted for estuarine and marine ecosystems. Marine pollution monitoring in the UK (e.g. as conducted by Cefas) is conducted to advance approaches to marine water quality monitoring as part of efforts to achieve good environmental status for marine waters. At present in the UK, this data is not yet as readily available as datasets in the EA WIMS portal, making the use of this data for mixture exposure and risk assessment difficult without committing significant additional resource and time to obtaining data and readying in for use.
3. Without access to marine specific datasets, understanding of the relative extent of exposure and risk between marine, estuarine and freshwater ecosystems should be treated as precautionary. In the EA WIMS dataset used here, the majority of available samples measured are for freshwaters (87%), followed by seawaters (11%) and estuaries (3%). Sites where potentially “toxic” mixtures were found were dominated by freshwater sites, being >99% of all cases. This suggests a higher-level mixture exposure in freshwaters, given the high percentage of “toxic” mixture sites compared to the overall proportion of such sites in the overall dataset. To test this assessment, more comprehensive assessment considering the data for marine ecosystems is needed. Within any such analysis, it is important to consider that chemical analysis considers pollutants linked to marine sources or known to be widely present in marine waters (e.g. antifoulants, microplastics). Estuarine environments in particular should



be a focus for any further assessment, given that the unique conditions leading to sedimentation may make them hotspots for pollution accumulation and, hence, mixture exposure.

4. Open access to systematic data on chemical occurrence in surface water can improve knowledge on which mixtures occur where and when. As we highlight in Question 1, regulatory agencies have begun to appreciate the value of making their chemical monitoring data available. Such data is invaluable for identifying the nature of environmental mixtures. There is as yet no single resource that researchers, regulator, industry or the public can visit to access the different types of water quality monitoring data available. Different datasets reside in different repositories. A hub that integrates other data (such as the Environment Agencies recent PFAS source inventory) would help to link mixture exposure drivers (e.g. land-use; topography; industrial, municipal, transport infrastructure), with climate and other stressor data to provide enhanced multi-stressor scenario assessments. Links with biodiversity data would further allow exposures to be linked to ecological effects.

#### *Component based mixture effect modelling for risk assessment*

Classical component-based mixture risk assessments require information on chemical exposure and hazard. Exposure information can come from chemical measurements made for relevant environmental samples, e.g. marine, estuarine or freshwater waters or sediments, or from fate modelling studies designed to predict environment concentrations in relevant media. The hazard term that is used for any risk assessment is derived from the available ecotoxicity data. In early studies, data for a single species, often a fish or cladoceran, were used. However, the growing availability of SSD data mean that values taken from these models are increasingly used. The growing range of measured and modelled exposure data and ecotoxicological hazard data are shaping a new state of the art for mixture risk assessment.

1. Mixture effect modelling for predictive and retrospective risk assessment applications requires information on mixture exposure and hazard for the range of chemicals present and species exposed. Previously many substances widely detected in the environment lacked reliable and robust information on their hazard, especially for emerging pollutants. Efforts to consolidate all available ecotoxicological data to allow development of SSDs for a much greater range of chemicals (Posthuma et al., 2019) or quantitative structure activity relationship models (Kar and Roy, 2010; Khan et al., 2019) have now expanded the range of substances for which usable hazard data is available. The recent publication of SSDs for >13,000 (Posthuma et al., 2019), represents a landmark advance in this space. It alone has provided an order of magnitude change to the number of substances that can be covered in an aquatic mixture risk assessment.
2. Although a significant advance, the new tranche of SSD information is not without issues and gaps. Posthuma et al. (2019) identified that some of the SSD models were based on more robust datasets than others. For example, some chronic SSDs were generated from multiple chronic toxicity datasets, while others are from chronic data derived from acute data by read across, and others from a combination of these data types. To reflect this, Posthuma et al. (2019) provided a quality score to their SSDs.



Conclusions made from risk assessment conducted using these SSDs would benefit from taking the uncertainty into account. Further, even though more comprehensive than before, gaps still exist, and some chemicals still have no SSD HC<sub>50</sub> values available. For example, when Spurgeon et al. (2022) used the SSDs of Posthuma et al. (2019) for mixture risk assessment using Environment Agency semi-quantitative GC-MS and LC-MS datasets, only ~80% of chemicals in the analytical suites had available HC<sub>50</sub> values, similar to the frequency found here when addressing Question 1. Notable substance classes with SSDs missing included 10 of the 13 measured PFAS and PCB congeners. To address these gaps, further efforts are needed to consolidate ecotoxicological data to maintain and grow the resources for overall, and even habitat specific, SSD generation. Quantitative structure activity relationship models could also be a route to gap filling.

3. To understand chemical hazard, HC<sub>50</sub> values provide a valuable resource. The toxicity datasets from which these SSDs are constructed are dominated by values for freshwater species. This freshwater focus means there is a potential to misrepresent the extent to which marine species may be at risk if, for example, there is evidence that marine species are systematically more sensitive than freshwater organisms. The comparative sensitivity of marine versus freshwater species has been addressed in a number of studies. These analyses have so far found predominantly similar (Kyriakopoulou et al., 2009; Yanagihara et al., 2022) or even lower (Wheeler et al., 2002) sensitivity for marine versus freshwater species. Based on these meta-analyses, there seems no reason to preclude the use of SSDs generated using data mainly for freshwater species because of systematic differences in sensitivities.
4. Mechanistic understanding lies at the heart of interpreting the effects of mixtures. Within their strict mechanistic context, the different additive models of CA and IA require knowledge of chemical mode of action. There are, however, challenges with such attribution. Mode of action can be species dependent, e.g. insecticides with similar modes of action in an insect may work differently, indeed differently from each other, in a non-insect species. The same may be true to two herbicides tested in an animal or microorganism. Because of this complexity, it may be impossible to truly use mechanisms for mixture model selection. Initial substances would have to rely on species “read across”, i.e. assuming that two herbicides that work by the same mechanism in a plant species also work by the same mechanism in an exposed invertebrate or microbe. Given the range of chemicals and species exposed in any environmental mixture scenario, it is unlikely that fully species-specific mechanistic mixture modelling will be available. Mechanistic knowledge for one or more chemical is always likely to be missing. This challenge means that by necessity, the choice of CA or IA for mixture modelling becomes a goal focussed or pragmatic decision. How the use of each model can be interpreted can instead be done based on their statistical concepts of potency additivity and effect probability. Use of both models simultaneously can provide a “prediction window” for the joint effect defined as the prediction space between the two model predictions. Practically, however, challenges in applying IA, e.g. the need for full dose-response data, often means that less data is needed and more conservative CA model is the only option used.
5. Complex mixtures of chemicals are present in UK surface waters. How complex these mixtures are viewed will depend on the threshold that is placed on deciding whether



a given chemical makes a substantive contribution to a mixture effect. Reports of mixture complexity are often based on detection limits, i.e. complexity = N of detected chemicals. Mixture effects can result when a large number of chemicals contribute a small amount to a mixture exposure; so called “something from nothing” effect (although actually a something big from lots of small somethings effect!) (Silva et al., 2002; Thrupp et al., 2018; Versieren et al., 2016). However, in many cases most of the biological effect comes from only quite a small number of the components present, with <5 substances dominating and the remainder contributing little to the mixture toxic pressure.

6. The lessons from laboratory ecotoxicology come mainly from studies of mixture of low (binary, ternary) complexity. The umbrella analysis to address Question 2 identified consistently the dominance of binary mixture studies (75% of all tests) compared to higher complexity mixtures. At first sight, this focus of laboratory testing on binary mixture seems inappropriate, given the potential complexity of mixtures in the field. However, as often few chemicals contribute a high proportion of the predicted mixture effect, lessons from binary or ternary mixture study, e.g. on frequency of additive and non-additive effects, magnitudes of synergism; and chemicals involved are likely to be more relevant than it seems at first sight.
7. The additive models of CA and IA originating from pre-second world war pharmacology, have a high predictive power for mixture effects. Although often used “stand-alone”, CA and IA can also be used within more complex models. These include 1) Combined CA and IA models that use CA initially to predict the joint effects of sets of similarly acting chemicals and IA to combine effects predictions for these groups (Kim et al., 2014; Qin et al., 2011); 2) Biotic Ligand, electrostatic uptake and ion-ion and ion-organism interactions (e.g. WHAM  $F_{Tox}$  models) which simulate interactions of ionic chemicals with the organism body surfaces to cause joint toxicity; 3) Multi-substance potentially affected fraction (msPAF) models which use CA (and IA) to predict the fraction of species affected by a mixture from individual chemical species sensitivity distributions. Use of these more complex models can overcome some of the conceptual problems in the use of additive models, by allowing assessments for chemical with similar and different modes of actions, accounting for environmental availability and linking mixture exposure to biological impacts.
8. Non-additive (synergistic or antagonistic) effects occur in tested mixtures. Models and tools exist to identify the mixture from observed versus additive predicted data (Cedergreen et al., 2012; Jonker et al., 2005). Where non-additive effects are identified, mechanistic attribution is plausible. For example, non-additive effects of metals have been linked to competitive effects that inhibit co-uptake across biological membranes to change environmental availability. Models that use chemical speciation and binding site completion theory and data to predict and incorporate the effects into joint effects models building on concepts, such as the biotic ligand model or WHAT F-Tox model, provide tools to develop these kinds of predictions. Other approaches that can identify mechanistic causes include *in vitro*, *in vivo* or *ex-vivo* assays able to quantify chemicals inhibition of key enzyme systems involved in xenobiotic metabolism that may cause one chemical to inhibit the breakdown of another (Dalhoff et al., 2020).



9. Legacy pollutants, e.g. metals, persistent organic pollutants, are highly represented in mixture exposures and are often identified in risk assessment as the most likely to cause biological effects. Because of the long pollution history of these chemicals, analytical methods and ecotoxicological data for such chemicals are well established making risk assessment easier. Emerging pollutants (e.g. pharmaceutical, plasticisers, household chemicals) appear less frequently as chemicals making a major contribution to mixture exposure. However, the data-sets for these chemicals are much smaller. As a result, the potential to include these classes of chemical in mixture risk assessment is more limited. There is also some mismatch between those pesticides measured, i.e. those listed as priority substances under the Water Framework Directive, and those in most widespread use. Regular review of analytical suites to ensure inclusion of emerging compounds is needed to maintain the relevance of monitoring data for timely mixture assessment.

*How mixtures effect prediction relate to impacts on ecosystems*

Prediction of mixture impacts aims to provide a quantitative assessment of the likelihood and scale of mixture effects on ecological communities. To understand whether this is the case, studies that measure chemical exposures and community responses are highly valued. Marine and other studies have looked to characterise species and community response to mixture exposures. The outcome from such studies inform both broad principles for assessment and promising avenues to move understanding further forward.

1. The current risk assessment approach is designed for chemical-by-chemical assessment using data for hazard assessment that draws largely from ecotoxicity tests that measure short-term, mainly acute, effects. These emphases mean that the effects of low level and long-term mixture exposure on marine and other species are not routinely considered in risk assessment. Landmark policy and economic changes have changed the profile of pollution that reaches the UK environment. Past point sources of pollution still provide a legacy problem. However, the focus is now turning more to more diffuse sources and emerging pollutants groups. Better analysis shows that many marine environments have pollutants present at measurable concentrations. That ecological communities at locations with the widest range of chemicals present often have lower biodiversity leads naturally to questions about the role that mixtures play in such changes.
2. Analysis of large-scale biodiversity datasets have produced an overall picture of biodiversity change in UK habitats over time. Assessment for freshwater biodiversity datasets for macroinvertebrate, fish and macrophytes generally produce a picture of improvement, e.g. family richness, over time (Pharaoh et al., 2024; Qu et al., 2023; Stockdale et al., 2014a). Such recoveries have been linked to improvement in water quality including declines in nutrient concentrations; lower biological oxygen demand and reduce toxic metal load. Despite positive trends, issues with freshwater quality remain. Even in those studies showing improvement, many sites are still harbour denude communities. Sites most likely to be affected by anthropogenic stress, such as catchments with a high proportion of agricultural land or urban area are more often

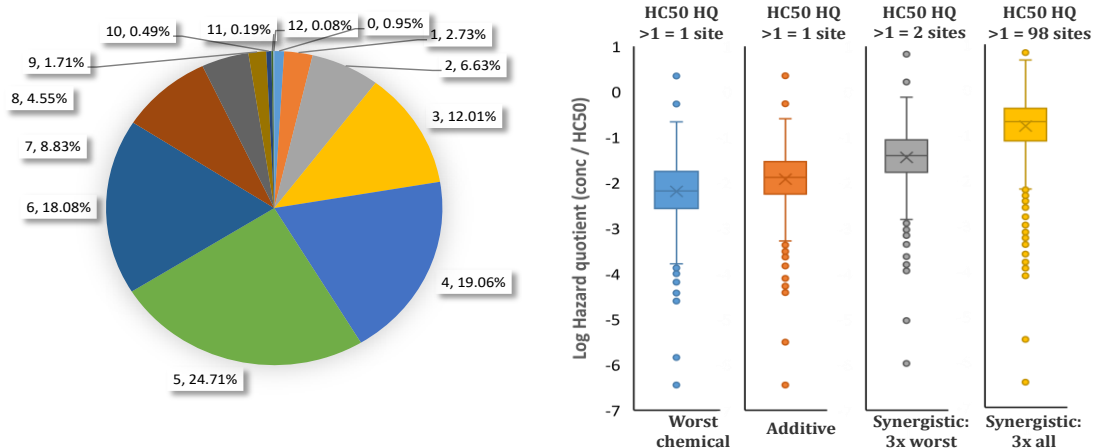


impacted (Pharaoh et al., 2024; Qu et al., 2023). These patterns suggest that while river quality has improved, it still remains unacceptably poor in local cases (Whelan et al., 2022). Long running marine biodiversity data exist for plankton, benthic invertebrate and fish communities. These surveys operate at both point, transect and national scale. The monitoring programs are not as well connected to sites used for pollution monitoring as for freshwater, where the Water Framework Directive acts as a stimulus for parallel water quality and biodiversity analysis. Trends in some marine surveys point to both biodiversity declines over time, e.g. for demersal communities, and also population increases, e.g. more increasing than declining fish species, with fishing, climate and pollution all implicated as causative factors (Mieszkowska et al., 2014).

3. Even in those cases where improvements have taken place, chemical pollutant exposure still cause impacts at multiple scales. Risk assessment frameworks have been in place for chemicals for decades. Yet these effects still occur. Why is this the case? This is not a simple question to answer. In some cases, impacts are the result of legacy pollution that would not now be authorised. However, even for new exposure chemicals and release scenarios effects still occur. Current ecotoxicological tests focus on simple measurement of vital rate, i.e. survival, reproduction, important for understanding population effect. However, there is growing evidence that chemical exposure can affect the metabolic, immunological and behavioural status of species in ways that can impact populations. To support better assessments, these novel endpoints need to be tractable for quantification as well as conceptualised within a modelling framework linking across molecular, cellular and individual scales to population impacts. A more mechanistic focus in marine and freshwater ecotoxicology would support such assessments.
4. Multiple case studies in marine and freshwater have investigated the effects of chemicals, primarily metals and pesticides on aquatic biodiversity. Chemical measurements by targeted and untargeted analysis provide information on the chemical concentrations at field sites. When included, mixture modelling usually relies on the CA approach, mainly because the data requirements for use of the IA cannot be met. Algae and benthic macroinvertebrates are the groups most assessed in such studies. Sites that had a high mixture toxicity pressure indicated a correlation with biodiversity loss. However, sites with lower biodiversity do not always have high concentrations of pollutants present, indicating that biodiversity loss results due to other environmental factors. Multivariate analysis of community structure association with environmental drivers, including chemical mixture pressure, provides a valuable approach for diagnosing the role of different pressures in ecological community change.
5. Synergism (or antagonism) as a response pattern to mixture exposure is not just of academic interest. Interactive effects leading to greater than expected toxicity of chemicals in a mixture can change the potential for biological effects from mixtures. Azole fungicides are a commonly identified group of synergistic chemicals. Assessment of EA surface water monitoring data from national monitoring (covering



analysis of >2,500 samples) indicates that azole chemicals released from fungicidal use in agriculture and medicine are commonly found in surface waters in England, with >4 azoles detected in >75% of samples (Figure 12). The impact of the presence of these azoles on the potential for the other chemicals to cause ecological impacts can be assessed by calculating risk in each sample for the most toxic chemical only, the mixture, and assuming azole effects cause synergism on the most toxic chemical or on all chemicals. For the most toxic chemical only, the median HQ across the >2,500 samples is two orders of magnitude lower than the effect threshold used for risk calculations (Figure 12 Right, Left hand bar). Mixture risk is marginally higher than for most toxic chemicals (Figure 12 Right, Centre left bar), accounting for 3-fold synergism increases median mixture risk to close to (Figure 12 Right, Centre right bar) and below (Figure 12 Left bar) one order of magnitude lower than the threshold for risk. Combined considering the mixture effect and allowing for synergism that can be mechanistically linked to the presence of azoles at even a low level of 3-fold effect together results in an ~30-fold increase in the level of predicted risk compared to the worst single chemical alone. For those chemicals that can be identified as common synergists, this could be as a reason for their greater regulatory management, including removals of authorisations for use.



**Figure 12.** Left: Number of agriculture and veterinary azoles present in Environment Agency measured surface water samples from England; Right: Risk of the most toxic chemical (Left hand bar); mixture risk (Centre left bar), single chemical risk with 3-fold synergism (Centre right bar) and mixture risk with 3-fold synergism (Right bar) for detected chemical in the same dataset.

- Regulatory inertia is a significant barrier to integrating the quite well-developed understanding of chemical mixture prevalence and potential for effects. The fear of the incomplete, such as an inability to predict synergism in all cases, often acts as a barrier to doing what will work in most cases, i.e. using additivity as an initial predictive model. As knowledge of the frequency/scale of synergism and its mechanistic

develop, it is feasible to integrate this knowledge into mixture assessments based initially on additivity.

*Advancing method for mixture effect detection and attribution*

A range of statistical and ecological diagnostic approaches can be used to confirm the role of chemical pollutants in effects on ecological communities. The use of such approaches is most valuable where they use mechanistic knowledge to ensure that the tools deployed are relevant to the chemicals and effects under assessment.

1. Ecotoxicology has grown as a largely observational science. As such, a mechanistic basis for understanding chemical effects has generally been underutilised. If we are to better understand ecotoxicological problems, we need to link cause-effect in a manner that the simple endpoint assays (survival, reproduction) used to meet regulatory needs fail to achieve. New mechanistic methods, such as the use of receptor linked biosensors (see Case Study 7 text) offer the possibility to use mechanistic knowledge of chemical impacts through different modes of action within an effect directed analysis approach. To date, this family of mechanistically focussed methods have not been widely considered for monitoring in the UK, but have been deployed for chemical exposure and effect prediction and diagnostics in EU and US research studies.
2. Genomic and transcriptomic data holds valuable information that help to improve assignment of mode of action in ecotoxicology to support mixture model selection and the identification of toxicokinetic or toxicodynamics interactions that can lead to non-additive effects. Insights from such data include information on species xenometabolic complexity; target receptor presence and structure, and cellular damage mitigation capacities. Despite significant efforts to develop relevant tools, omic resources remain largely underutilised and are not commonly integrated into hazard assessments. This underutilisation is a significant oversight, especially as projects such as the Darwin Tree of Life project ([www.darwintreeoflife.org/](http://www.darwintreeoflife.org/)) are vastly increasing the availability of genomic data for UK environmental species (with the ultimate plan to sequence all UK eukaryotes). This effort will offer a huge resource with the potential to transform predictive ecotoxicology (as well as other ecological areas) by providing a more mechanistic underpinning to previously observational fields.
3. The adverse outcome pathways (AOP) concept is designed as a mechanistic framework for use in ecotoxicological research and diagnostics. An AOP takes the form of a quantitative or qualitative model that links the initial interactions of the chemical with the biological target (the molecular initiating event), through effect at different levels of biological organisation, to toxicity on apical endpoints. A centralised repository of adverse outcome pathway models, the AOP Wiki ([aopwiki.org/](http://aopwiki.org/)), is designed to share this mechanistic knowledge. At the time of writing 481 models are included in this resource. Many of these are currently in draft form. As such, they provide only a hypothetical summary of the links between exposure and effect. Even such draft models offer a useful resource to identify the most plausible biological measure that can be made to identify whether a particular chemical exposure is



playing a role in an observed toxic effect. This kind of informed assessment has a greater diagnostic potential than taking a mechanistically agnostic approach.

4. There is a long history of the use of biomarkers for pollution effect detection in marine ecosystems. There is a decade or more of evidence that the use of biomarkers can play a useful role in the detection of chemical exposure and the physiological and ecological consequences of single chemical and mixture exposure (Lionetto et al., 2021). Long-term programmes like the UK Clean Seas Environmental Monitoring Programme have used biological effects techniques to investigate acetylcholinesterase inhibition, cytochrome P45 activity and the presence of biliary PAH metabolites for detecting chemical exposure in fish. Regional patterns of response indicate potential exposure to substances that affect the biomarkers, e.g. organophosphates and carbamates for acetylcholinesterase inhibition. However, care is needed to interpret such results at individual sites, as biological factors, such as sex and organism age and condition can affect results (Dalessandri et al., 2023).
5. Biomarker use only provides diagnostic value if the biological effect investigated is appropriate for the chemicals present. For example, acetylcholinesterase activity can detect exposure to chemicals, like organophosphate and carbamate insecticides, then inhibit this enzyme, but not other chemicals. Use of a greater range of biomarker methods, deployed as a battery increases the potential to identify biologically active chemicals in an environmental mixture. Similar strategies to those used to develop and verify the relevance of human biomarkers can be used to identify biomarker toolkit components (Owen et al., 2008).
6. The use of biosensors for detecting chemical stressors has already been demonstrated at scale in a US based research study (Blackwell et al., 2019). The high throughput nature of biosensor methods allows the measurement of effects against these pathways. Measurement of the chemicals present in waters and sediments at any investigated sites, alongside the assessment of biological indicator responses provide valuable support to diagnose the causes of the identified receptor activations.
7. The risk of synergism can also be addressed by taking a mechanistic perspective. The reviews of experimental studies indicate that the largest cases of synergism occur when one chemical interferes with the detoxification of a second. *In vitro*, *in vivo* and *ex-vivo* assay can be used to screen chemicals for the potential to cause such effects, e.g. the inhibition of cytochrome P450 enzymes. The potential for these chemicals to affect pathways resulting in synergism should be viewed as a specific case of risk in water management that may call for greater regulatory management action for the chemicals involved.
8. Accelerating development in mechanistic toxicology arising from innovations in omics and biotechnology will continue to provide new and promising tools for the detection and assessment of chemical mixture effects. Identifying the diagnostic reality from premature hyperbole is necessary to identify the most valuable and applicable of available tools. The adverse outcome pathways approach provides a generalised



mechanistic framework in which the links between measure of effect at different levels of biological organisation can be placed to support such identification.

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**Appendix 1:** Chemicals with EA WIMS monitoring and SSD data. An effect threshold (10<sup>th</sup> HC<sub>50</sub>) has been derived from the chemical SSD data. Quality scores for each chemical SSD from Posthuma et al (2019) are added, showing highest (“1111”) to lowest (“4444”) quality of the SSDs constructed. The scores are comprised on 4-digits which denote to the slope of the curve (digit 1), taxonomic group coverage (digit 2), data origin (digit 3) and data-bridging techniques (digit 4). (-) Refers to chemicals in the EA WIMS monitoring data with no SSD data.

Chemical	Chemical group	10 <sup>th</sup> HC <sub>50</sub> (µgL <sup>-1</sup> )	SSD quality score
(1 alpha,2 alpha,3 beta,4 alpha,5 beta,6 beta)1,2,3,4,5,6-Hexachlorocyclohexane	Pesticides	138.04	1211
(1aR,2R,2aS,3S,6R,6aR,7S,7aS)-rel-3,4,5,6,9,9-Hexachloro-1a,2,2a,3,6,6a,7,7a-octahydro-2,7:3,6-dimethanonaphth[2,3-b]oxirene	Pesticides	2.34	1111
[(4-Aminophenyl)sulfonyl]carbamic acid, Methyl ester	Pesticides	30902.95	1111
(Aminomethyl)phosphonic acid	Pesticides	19952.62	1311
(R)-N-Ethyl-2-[[[(phenylamino)carbonyl]oxy]propanamide	Pesticides	17782.79	1311
1,1'-(2,2,2-Trichloroethylidene)bis(4-chlorobenzene)	Pesticides	2.51	1111
1,1'-(2,2-Dichloroethenylidene)bis[4-chlorobenzene]	Pesticides	8.32	1111
1,1,1-Trichloroethane	POPs	9772.37	1111
1,1-Dichloroethene	POPs	15488.17	1111
1,1'-Oxybis[2,4-dibromobenzene]	POPs	1047.13	2411
1,2,3,4,10,10-Hexachloro-1,4,4a,5,8,8a-hexahydro-(1a,4a,4ab,5a,8a,8ab)-1,4:5,8-dimethanonaphthalene	Pesticides	5.25	1111
1,2,3,4,5,6-Hexachloro((1a,2b,3a,4b,5a,6b)cyclohexane	Pesticides	85.11	1325
1,2,3,4,5,6-Hexachloro(1a,2a,3a,4b,5a,6b)cyclohexane	Pesticides	154.88	1311
1,2,3,4,5-Pentachlorobenzene	Pesticides	97.72	1111
1,2,3-Trichlorobenzene	Pesticides	229.09	1111
1,2,4,5-Tetrachloro-3-nitrobenzene	Pesticides	56.23	1111
1,2,4-Trichlorobenzene	Pesticides	295.12	1111
1,2,4-Trimethylbenzene	Pesticides	478.63	1111
1,2-Dichlorobenzene	Pesticides	870.96	1111
1,2-Dichloroethane	POPs	16982.44	1111
1,2-Dichloropropane	POPs	10000	1111
1,2-Ethanediol	POPs	1513561.25	1111
1,3,5-Trichlorobenzene	POPs	416.87	1211
2-(2,4,5-Trichlorophenoxy)acetic acid	Pesticides	2041.74	1111



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2-(4,5-Dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl)-3-pyridinecarboxylic acid	Pesticides	5754.4	1211
2,2',4,4',5,5'-Hexachloro-1,1'-biphenyl	POPs	0.13	2411
2,2',4,5,5'-Pentachloro-1,1'-biphenyl	POPs	2.75	1311
2,2',5,5'-Tetrachloro-1,1'-biphenyl	POPs	3.02	2411
2,2'-oxydiethanol	POPs	2238721.14	1111
2,3,5,6-Tetrachlorobenzeneamine	Pesticides	26.92	2411
2,3,6-Trichlorobenzoic acid	Pesticides	0.9	2411
2,3-Dimethylphenol	POPs	1230.27	1211
2,4,4'-Trichloro-1,1'-biphenyl	POPs	15.85	2411
2,4,6-Trichlorophenol	POPs	263.03	1111
2,4-D	Pesticides	5128.61	1111
2,4-DB	Pesticides	1584.89	1211
2,4-Dichlorophenol	POPs	707.95	1111
2,4-Xylenol	POPs	630.96	1111
2,5-Dichlorophenol	POPs	316.23	1111
2,5-xylenol	POPs	812.83	1211
2,6-Dichlorophenol	POPs	933.25	1111
2,6-Xylenol	POPs	707.95	1111
2-Chlorophenol	POPs	1621.81	1111
2-ethoxy-2-methylpropane	POPs	20417.38	1311
2-Methyl-2-(methylsulfinyl)propionaldehyde, O-(Methylcarbamoyl)oxime	POPs	4.27	1311
3,4-Dimethylphenol	POPs	977.24	1111
3,5-xylenol	POPs	1621.81	1111
3-Chlorophenol	POPs	1096.48	1111
4-(1,1,3,3-Tetramethylbutyl)phenol	POPs	20.89	1111
4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid	Pesticides	2398.83	1111
4-Chloro-3,5-dimethylphenol	POPs	107.15	1111
4-chloro-3-methylphenol	POPs	616.6	1111
4-chloro-o-cresol	POPs	288.4	1111
4-Chlorophenol	POPs	954.99	1111
4-CPA	POPs	4786.3	1336
4-Methyl-2-pentanone	Organic solvents	104712.85	1211
4-Nonylphenol	POPs	19.5	1111
6-Chloro-N-(1-methylethyl)-1,3,5-triazine-2,4-diamine	Pesticides	1.74	1325
6-Chloro-N-ethyl-1,3,5-triazine-2,4-diamine	Pesticides	891.25	2411
Acenaflyleen	Pesticides	158.49	1111
Acenaphthene	PAHs	91.2	1111
Acetone	Organic solvents	398107.17	1111
Aclonifen	Pesticides	19.5	1211
Aluminum	Metal/metalloids	199.53	1111



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Amoxicillin	Pharmaceuticals	7413.1	1111
Anthracene	PAHs	1.86	1111
Antimony	Metal/metalloids	2041.74	1211
Arsenic	Metal/metalloids	245.47	1111
Atrazine	Pesticides	190.55	1111
Azoxystrobin	Pesticides	44.67	1111
Barium	Metal/metalloids	22908.68	1311
Benazolin	Pesticides	12589.25	1111
Bentazone	Pesticides	13182.57	1111
Benzene	PAHs	7585.78	1111
Benzo(a)Anthracene	PAHs	0.41	1111
Benzo(a)pyreen	PAHs	3.02	1111
Benzo(b)fluorantheen	PAHs	0.37	1122
benzo(ghi)perylene	PAHs	0.05	1122
Benzo(k)fluorantheen	PAHs	0.94	1122
beta-Endosulfan	Pesticides	2.14	1311
Bifenox	Pesticides	229.09	1211
Bis(2-ethylhexyl) phthalate	Phthalates	426.58	1111
Boron	Metal/metalloids	9120.11	1211
Bromodichloromethane	POPs	23988.33	1311
Bromoxynil	Pesticides	295.12	1111
Cadmium	Metal/metalloids	81.28	1111
Carbendazim	Pharmaceuticals	338.84	1111
Chloorthalonil	Pesticides	10.23	1111
Chlorfenvinphos	Pesticides	13.18	1111
Chloridazon	Pesticides	1995.26	1211
Chlorobenzene	PAHs	1479.11	1111
Chlorotoluron	Pesticides	1023.29	1111
Chlorotriphenylstannane	Organic solvents	0.57	1111
Chlorpropham	Pesticides	478.63	1211
Chlorpyrifos	Pesticides	1.12	1111
Chromium	Metal/metalloids	776.25	1111
Chrysene	PAHs	125.89	1111
Cis-Permethrin	Pesticides	0.7	1111
Clopyralid	Pesticides	6760.83	1111
Cobalt	Metal/metalloids	416.87	1211
Copper	Metal/metalloids	18.2	1111
Cyanide	Pesticides	128.82	1311
Cypermethrin   Zeta-cypermethrin	Pesticides	0.08	1111
Deltamethrin	Pesticides	0.2	1111
Desmetryn	Pesticides	676.08	1211
Diazinon	Pesticides	21.88	1111
Dibenz(a,h)anthracene	PAHs	0.11	1122
Dibromochloromethane	Pesticides	4677.35	1211
Dibutyltin dilaurate	POPs	95.5	2411
Dicamba	Pesticides	2691.53	1111



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Dichlobenil	Pesticides	933.25	1111
Dichloromethane	Organic solvents	25118.86	1111
Dichlorprop	Pesticides	812.83	1111
Dichlorvos	Pesticides	33.11	1111
Dimethoate	Pesticides	204.17	1111
Dimethylbenzene	POPs	3890.45	1111
Diuron	Pesticides	66.07	1111
Endosulfan	Pesticides	0.91	1111
Estradiol	Pharmaceuticals	2.29	1122
Estrone	Pharmaceuticals	1.29	1324
Ethinylestradiol	Pharmaceuticals	190.55	1111
Ethofumesate	Pesticides	2238.72	1211
Ethylbenzene	POPs	1737.8	1111
Fenitrothion	Pesticides	15.85	1111
Fenuron	Pesticides	10715.19	1211
Fluoranthene	PAHs	13.49	1111
Fluorene	PAHs	239.88	1111
Fluroxypyr	Pesticides	3981.07	1111
Glyphosate	Pesticides	4265.8	1111
Hexabromocyclododecane	POPs	1.32	1111
Hexachlorobenzene	POPs	79.43	1111
Hexachlorobutadiene	POPs	40.74	1111
Hexamethyl phosphoramidate	POPs	524807.46	1311
indeno(123cd)pyrene	PAHs	1.2	1122
Iron	Metal/metalloids	3090.3	1211
Isodrin	Pesticides	0.85	2411
Isoproturon	Pesticides	87.1	1111
Lead	Metal/metalloids	354.81	1111
Lindane	Pesticides	11.75	1111
Linuron	Pesticides	64.57	1111
Lithium	Metal/metalloids	4466.84	1311
Malathion	Pesticides	28.18	1111
Manganese	Metal/metalloids	3162.28	1211
MCPA	Pesticides	2884.03	1111
m-Cresol	POPs	2884.03	1111
Mecoprop	Pesticides	4677.35	1311
Mercury	Metal/metalloids	18.2	1111
Mesitylene	POPs	1318.26	1111
Metaldehyde	Pesticides	8128.31	1311
Metazachlor	Pesticides	630.96	1211
Methiocarb	Pesticides	22.91	1111
Metoxuron	Pesticides	11481.54	1111
Metsulfuron-methyl	Pesticides	436.52	1111
Molybdenum	Metal/metalloids	38904.51	1111
Monolinuron	Pesticides	323.59	1111
Monuron	Pesticides	3388.44	1111



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Naphthalene	PAHs	416.87	1111
Neburon	Pesticides	87.1	1211
N-Ethyl-1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-heptadecafluoro-1-octanesulfonamide	PFASs	117.49	1311
Nickel	Metal/metalloids	239.88	1111
O-Cresol	POPs	3019.95	1111
Octamethylphosphoramidate	Pesticides	16982.44	1211
O-xylene	PAHs	1096.48	1111
P-Cresol	POPs	1230.27	1111
P-cymene	PAHs	2137.96	1111
Pendimethalin	Pesticides	95.5	1111
Pentachlorophenol	POPs	40.74	1111
Pentadecafluorooctanoic acid	PFASs	4265.8	1311
perfluorooctansulfonaat (PFOS)	PFASs	2290.87	1111
Permethrin	Pesticides	1.2	1111
Phenanthrene	PAHs	46.77	1111
Phenol	POPs	5623.41	1111
Prometryn	Pesticides	107.15	1111
Propazine	Pesticides	1000	1211
Propoxur	Pesticides	75.86	1111
Propyl benzene	POPs	177.83	1111
Propyzamide	Pesticides	3162.28	1111
Pyrene	PAHs	8.91	1111
Rotenone	Pesticides	17.38	1111
Selenium	Metal/metalloids	691.83	1111
Simazine	Pesticides	758.58	1111
Styrene	Plastic polymers	1778.28	1111
Terbutryn	Pesticides	29.51	1111
Tert-butyl methyl ether	POPs	74131.02	1111
Tetrabutylstannane	POPs	33.11	1111
Tetrachloroethylene	POPs	1905.46	1111
Tetrahydrofuran	POPs	380189.4	1311
Tetraphenylstannane	POPs	0.97	1322
Titanium	Metal/metalloids	2454.71	1311
Toluene	POPs	4466.84	1111
Trans-1,2-Dichloroethylene	POPs	21877.62	1311
trans-3-(2,2-Dichloroethyl)-2,2-dimethylcyclopropanecarboxylic acid, (3-Phenoxyphenyl)methyl ester	Pesticides	1.32	1111
Tri-allate	Pesticides	64.57	1111
Tribromomethane	Organic solvents	1621.81	1111
Tributyltin	Pesticides	1.41	1111
Trichloroethylene	POPs	5888.44	1111
Trichloromethane	POPs	6918.31	1111
Triclopyr	Pesticides	363.08	1111
Triclosan	Pharmaceuticals	11.22	1111
Trietazine	Pesticides	229.09	1336



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Uranium	Metal/metalloids	61.66	1111
Vanadium	Metal/metalloids	186.21	1111
Vinyl chloride	Plastic polymers	28183.83	1111
Zinc	Metal/metalloids	165.96	1111
11DCIEthan	Other	(-)	(-)
c12DCIEthe	Other	(-)	(-)
Trihalomethn	Other	(-)	(-)
11DiClPropen	Other	(-)	(-)
PCB Con 180	PCBs	(-)	(-)
PCB Con 138	PCBs	(-)	(-)
PCBs	PCBs	(-)	(-)
PCB Con 118	PCBs	(-)	(-)
FOSA	PFAS	(-)	(-)
PFEEESA	PFAS	(-)	(-)
PFdodencAcid	PFAS	(-)	(-)
MeFOSAA-B	PFAS	(-)	(-)
2-Ethylphenl	Other	(-)	(-)
SecButylbenz	Other	(-)	(-)
7:3 FTCA	PFAS	(-)	(-)
PFtetdncAcid	PFAS	(-)	(-)
HFPO-DA	Other	(-)	(-)
PFNS	PFAS	(-)	(-)
3:3 FTCA	PFAS	(-)	(-)
9Cl-PF3ONS	Other	(-)	(-)
DiClBenzPhWW	Other	(-)	(-)
8:2 FTSA	PFAS	(-)	(-)
1,2,3-Trimet	Other	(-)	(-)
6:2 FTSA	PFAS	(-)	(-)
PFHpS	PFAS	(-)	(-)
PFDoS	PFAS	(-)	(-)
4:2 FTSA	PFAS	(-)	(-)
PFDS	PFAS	(-)	(-)
ADONA	PFAS	(-)	(-)
t-Hept Epox	Other	(-)	(-)
TrCFMethan	Other	(-)	(-)
5:3 FTCA	PFAS	(-)	(-)
MeFOSAA-L	PFAS	(-)	(-)
PFUnDS	PFAS	(-)	(-)
PFecHS	PFAS	(-)	(-)
PFTTrDA	PFAS	(-)	(-)
PFundencAcid	PFAS	(-)	(-)
N-MeFOSA	PFAS	(-)	(-)
EtFOSAA-L	PFAS	(-)	(-)
PFnonncAcid	PFAS	(-)	(-)
PFMOBA	PFAS	(-)	(-)
PFMOPrA	PFAS	(-)	(-)



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B-[e]-pyrene	PAHs	(-)	(-)
PFdecncAcid	PFAS	(-)	(-)
Perylene	Other	(-)	(-)
11Cl-PF3OUdS	Other	(-)	(-)
PFPeS	PFAS	(-)	(-)
EtFOSAA-B	PFAS	(-)	(-)
NFDHA	PFAS	(-)	(-)
Cr Hex Filt	Other	(-)	(-)
S-A-T-azine	Other	(-)	(-)
4Phenoxybutyr	Other	(-)	(-)
2Phenoxyprop	Other	(-)	(-)
MCPB	Other	(-)	(-)
PFHxSA	PFAS	(-)	(-)
PFBA	PFAS	(-)	(-)
PFheptncAcid	PFAS	(-)	(-)
FBSA	PFAS	(-)	(-)
PFBS	PFAS	(-)	(-)
PFHxS-B	PFAS	(-)	(-)
PFHxS-L	PFAS	(-)	(-)
2,3,5,6-TCIT	Other	(-)	(-)
PCB 149	PCBs	(-)	(-)
PCB 126	PCBs	(-)	(-)
TDE (OP)	Other	(-)	(-)
PCB Con 035	PCBs	(-)	(-)
HCH Epsilon	Other	(-)	(-)
PCB Con 105	PCBs	(-)	(-)
PCB Con 31	PCBs	(-)	(-)
PCB Con 156	PCBs	(-)	(-)
PCB Con 169	PCBs	(-)	(-)
PCB Con 170	PCBs	(-)	(-)
PFpentncAcid	PFAS	(-)	(-)
PFhexncAcid	PFAS	(-)	(-)
PhenoxytcAcid	Other	(-)	(-)
EtFOSE	PFAS	(-)	(-)
MeFOSE	PFAS	(-)	(-)
StrontumFilt	Metal/metalloids	(-)	(-)
Strontium-Sr	Metal/metalloids	(-)	(-)
PBDE 154	PBDEs	(-)	(-)
PBDE 85	PBDEs	(-)	(-)
PBDE 100	PBDEs	(-)	(-)
PBDE 66	PBDEs	(-)	(-)
PBDE 183	PBDEs	(-)	(-)
PBDE 138	PBDEs	(-)	(-)
PBDE 99	PBDEs	(-)	(-)
PBDE 28	PBDEs	(-)	(-)
PBDE 153	PBDEs	(-)	(-)



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PBDE Total	PBDEs	(-)	(-)
Dtgt Cat BAC	Other	(-)	(-)
DtrgtAncSyn	Other	(-)	(-)
Dtrgt NncSyn	Other	(-)	(-)
Sodium - Na	Metal/metalloids	(-)	(-)
Tot Drins(4)	Other	(-)	(-)
PAHs	PAHs	(-)	(-)
PirimiphsEth	Other	(-)	(-)
NDPA	Other	(-)	(-)
2CINaphthaln	Other	(-)	(-)
DnOcPhthalte	Other	(-)	(-)
Phenols Mono	Other	(-)	(-)
PFHxDA	PFAS	(-)	(-)
HFPO-TA	Other	(-)	(-)
PFTrDS	PFAS	(-)	(-)
DiOctyl Sn	Other	(-)	(-)
DtrgtAnc&Nnc	Other	(-)	(-)
2CI5NToluene	Other	(-)	(-)
SCCPs	Other	(-)	(-)
Na- Filtered	Metal/metalloids	(-)	(-)



**Appendix 2.** Case studies of the field assessment of mixture exposure and linked to modelled or quantified effects in marine, estuarine and freshwater ecosystems

**Case study 1.** Relationships between mixture pressure and biodiversity

<p>Ginebreda, A., Kuzmanovic, M., Guasch, H., de Alda, M.L., López-Doval, J.C., Muñoz, I., Ricart, M., Romani, AM., Sabater, S; Barceló, D. (2014). <b>Assessment of multi-chemical pollution in aquatic ecosystems using toxic units: Compound prioritization, mixture characterization and relationships with biological descriptors.</b> Science of the total environment, 468-469, 715-713</p>	
<b>Location</b>	Spain
<b>Waterbody type</b>	River
<b>Landuse surrounding area</b>	Agricultural, industrial (tannery, textile and paper industries) and urban
<b>Sampling information</b>	Multiple sites (n = 4) along the river across a pollution gradient
<b>Chemicals measured</b>	Pharmaceuticals (n = 29) and pesticides (n = 22). Only 16 of the 22 pesticides and 19 of the 29 pharmaceuticals were measured above LoD and included in mixture model.
<b>Aquatic species</b>	Diatoms (algae) and Daphnia (macroinvertebrates)
<b>Biological metrics</b>	<b>Algal biofilm:</b> chlorophyll-a content, photosynthetic capacity (using Ymax) and diatom community assemblage (using diatom PC_1 ordination species data). <b>Benthic macroinvertebrates:</b> species diversity (using Shannon-Wiener index) and biomass.
<b>Toxicity data used for model</b>	EC <sub>50</sub> toxicity data for diatoms and daphnia from published literature were used in mixture model.
<b>Mixture model</b>	Concentration Addition
<b>Comparison between predicted mixture effects and field observations</b>	<b>Algal biofilm:</b> Moderate inverse correlation between diatom diversity and sum of toxic units for algae ( $\sum TU_{algae}$ ) and direct correlation between photosynthetic capacity and $\sum TU_{algae}$ . <b>Benthic macroinvertebrates:</b> Good inverse correlation between macroinvertebrate biodiversity and $\sum TU_{daphnia}$ and direct correlation between macroinvertebrate biomass and $\sum TU_{daphnia}$ .
<b>Key biological findings</b>	<b>Algal biofilm:</b> Species at polluted sites disappear and community simplifies to a



	<p>smaller number of more tolerant species, resulting in a decrease in diversity.</p> <p><b>Benthic macroinvertebrates:</b> Sensitive species at polluted sites disappear and community simplifies to a smaller number of more tolerant species, resulting in a decrease in diversity. However, for biomass, the tolerant species benefit from the decrease in interspecific competence and higher quantity of organic matter for feeding.</p>																																																
<p><b>Other findings</b></p>	<ul style="list-style-type: none"> <li>- At contaminated sites the toxic load was dominated by a few compounds.</li> <li>- The contribution of pharmaceuticals to the toxic load increases in urban areas.</li> <li>- At cleaner sites, more compounds contributed to the toxic load.</li> <li>- Sites reflected a downstream pollution gradient corresponding to loss of ecological quality.</li> </ul>																																																
<p><b>Plots of key results</b></p> <p>Shows increasing trend in toxic units (TUs) for (a) algae and (b) daphnia in each sampling site along the pollution gradient. Sites A2 and LL4 were the most polluted sites along the river, being dominated by pharmaceuticals pesticides respectively. Sites A1 and LL1 are less polluted upstream areas. Sites LL2, LL3 and A3 are within urban areas with wastewater input.</p>	<p><b>a</b></p> <table border="1"> <caption>Data for Plot (a): Algae</caption> <thead> <tr> <th>Site</th> <th><math>\mu(\text{LnTU units})</math></th> <th><math>\sigma(\text{LnTU units})</math></th> </tr> </thead> <tbody> <tr><td>A1</td><td>-13.2</td><td>3.6</td></tr> <tr><td>LL2</td><td>-13.0</td><td>3.2</td></tr> <tr><td>LL1</td><td>-12.5</td><td>3.0</td></tr> <tr><td>LL3</td><td>-12.3</td><td>3.4</td></tr> <tr><td>A3</td><td>-12.5</td><td>3.7</td></tr> <tr><td>A2</td><td>-11.5</td><td>3.8</td></tr> <tr><td>LL4</td><td>-11.2</td><td>3.7</td></tr> </tbody> </table> <p><b>b</b></p> <table border="1"> <caption>Data for Plot (b): Daphnia</caption> <thead> <tr> <th>Site</th> <th><math>\mu(\text{LnTU units})</math></th> <th><math>\sigma(\text{LnTU units})</math></th> </tr> </thead> <tbody> <tr><td>A1</td><td>-16.0</td><td>3.6</td></tr> <tr><td>LL1</td><td>-14.0</td><td>3.5</td></tr> <tr><td>LL2</td><td>-14.5</td><td>3.7</td></tr> <tr><td>LL3</td><td>-13.8</td><td>3.8</td></tr> <tr><td>A3</td><td>-13.5</td><td>4.2</td></tr> <tr><td>A2</td><td>-12.8</td><td>4.4</td></tr> <tr><td>LL4</td><td>-12.5</td><td>4.5</td></tr> </tbody> </table>	Site	$\mu(\text{LnTU units})$	$\sigma(\text{LnTU units})$	A1	-13.2	3.6	LL2	-13.0	3.2	LL1	-12.5	3.0	LL3	-12.3	3.4	A3	-12.5	3.7	A2	-11.5	3.8	LL4	-11.2	3.7	Site	$\mu(\text{LnTU units})$	$\sigma(\text{LnTU units})$	A1	-16.0	3.6	LL1	-14.0	3.5	LL2	-14.5	3.7	LL3	-13.8	3.8	A3	-13.5	4.2	A2	-12.8	4.4	LL4	-12.5	4.5
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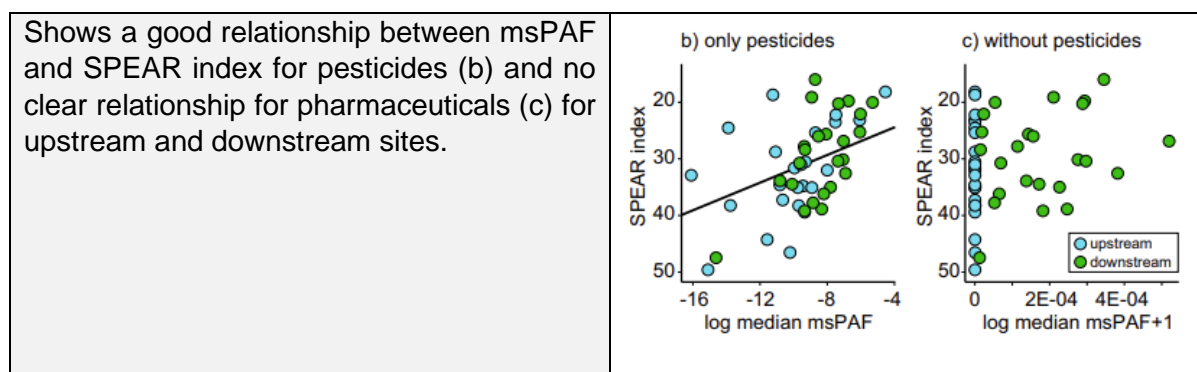
**Case study 2.** Relationships between mixture pressure and biodiversity

<p>Munz, N.A., Burdon, F.J., de Zwart, D., Junghans, M., Melo, L., Reyes, M., Schönenberger, U., Singer, HP., Spycher, B., Hollender, J., Stamm, C. (2017). <b>Pesticides drive risk of micropollutants in wastewater-impacted streams during low flow conditions.</b> Water Research, 110, 366-377</p>	
<p><b>Location</b></p>	<p>Switzerland</p>



<b>Waterbody type</b>	River
<b>Landuse surrounding area</b>	Urban, arable, meadows and woodland
<b>Sampling information</b>	Grab samples taken from upstream, downstream and effluent waters of 24 WWTPs.
<b>Chemicals measured</b>	Pesticides and pharmaceuticals (n = 389) and metals (Ag, Cd, Cr, Co, Cu, Fe, Mn, Ni, Pb and Zn).
<b>Aquatic species</b>	Macroinvertebrates
<b>Biological metrics</b>	Macroinvertebrate community/assemblages (species absence and presence) used to calculate trait-based species at risk (SPEAR) index. This index is indicative of pesticide contamination based on presence or absence of taxa sensitive to pesticides.
<b>Toxicity data used for model</b>	Acute EC <sub>50</sub> toxicity data from a database from a published study
<b>Mixture model</b>	Concentration Addition
<b>Comparison between predicted mixture effects and field observations</b>	Good agreement with observed biological effects on macroinvertebrates where pesticides seemed to be the main drivers of risk at both upstream and downstream locations. Significant correlations found between msPAF and SPEAR index across the 24 sites for pesticides. No significant correlation found for pharmaceuticals.
<b>Key biological findings</b>	Limited toxicity data available for pharmaceuticals compared to pesticides could be an explanation for why no significant correlation is found between multi-substance potentially affected fraction (msPAF) and SPEAR index across the 24 sites for pharmaceuticals.
<b>Other findings</b>	<ul style="list-style-type: none"> <li>- Over the 24 sites, acute toxicity pressure on aquatic species (msPAF) is relatively low (0-2.1%) and below the 5% affected threshold used to assess species risk.</li> <li>- Macroinvertebrate communities downstream were influenced by discharged wastewater.</li> <li>- Acute toxic pressure was mainly driven by pesticides (apart from the anti-inflammatory drug diclofenac).</li> <li>- Only a few substances explained most of the total risk, e.g. diclofenac, diazinon and clothianidin.</li> </ul>
<b>Plots of key results</b>	





### Case study 3. Relationships between mixture pressure and biodiversity

<p>Pereira, A.S., Dâmaso-Rodrigues, M.L., Amorim, A., Daam, M.A., Cerejeira, M.J. (2018). <b>Aquatic community structure in Mediterranean edge-of-field waterbodies as explained by environmental factors and the presence of pesticide mixtures.</b> <i>Ecotoxicology</i>, 27, 661-674.</p>	
<b>Location</b>	Portugal
<b>Waterbody type</b>	Irrigation water ditches adjacent to maize and tomato crop areas
<b>Landuse surrounding area</b>	Agricultural
<b>Sampling information</b>	Surface water (n=54) samples collected from 6 sites
<b>Chemicals measured</b>	Pesticides (n=19) (8 herbicides, 8 insecticides and 3 fungicides)
<b>Aquatic species</b>	Phytoplankton and macroinvertebrates
<b>Biological metrics</b>	<b>Algae:</b> Taxa abundance data and chlorophyll-a measurements <b>Macroinvertebrates:</b> Taxa abundance data
<b>Toxicity data used for model</b>	LC <sub>50</sub> toxicity data (mortality endpoints) from US EPA ECOTOX and E-toxBase databases.
<b>Mixture model</b>	Response Addition (i.e., Independent Action)
<b>Comparison between predicted mixture effects and field observations</b>	<b>Algae:</b> No clear relationship between taxa abundance and msPAF values <b>Macroinvertebrates:</b> Good comparison between taxa abundance and msPAF values
<b>Key biological findings</b>	<b>Algae:</b> Community shifts observed across sites with different predicted msPAF. Diatoms and blue-green algae highly dominated taxa abundances and the presence of green algae could be associated with sample locations with lower values of msPAF. <b>Macroinvertebrates:</b> Taxa abundance in samples with lower msPAF values was



	higher than in areas with higher msPAF values.
<p><b>Other findings</b></p>	<ul style="list-style-type: none"> <li>- The variance seen in ecosystem communities is highly influenced by habitat and physical-chemical conditions.</li> <li>- Toxicity of the mixture was mostly driven by only a few pesticides.</li> <li>- Pesticide mixture toxicity explained 23.7% of variance in phytoplankton and macroinvertebrate species abundances.</li> <li>- Sites with higher mixture pressure were associated with a decrease in pesticide vulnerable species and decrease in taxonomic diversity.</li> <li>- Pesticides which contributed the most to species loss were chlorpyrifos (35%), cypermethrin (21%) and cyalothrin (10%) for all samples.</li> </ul>
<p><b>Plots of key results</b> Redundancy ordination analysis (RDA) of macroinvertebrate and algal species data and msPAF values. Shows that taxa abundance is generally lower at sites with higher msPAF (predicted proportion of species impacted)</p>	

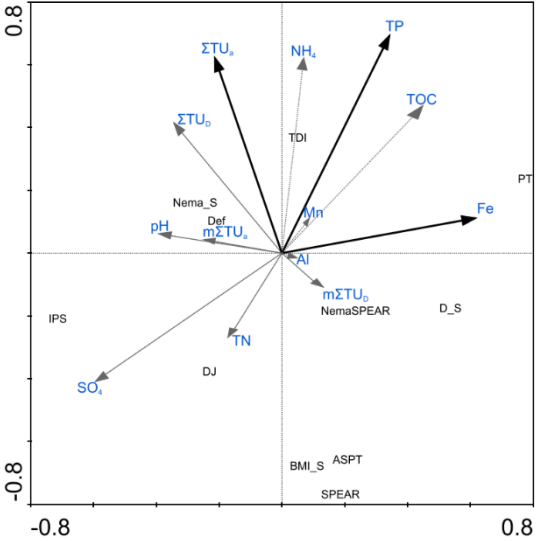
**Case study 4. Relationships between mixture pressure and biodiversity**

<p>Bighiu, MA., Höss, S., Traunspurger, W., Kahlert, M. Goedkoop, W. (2020) <b>Limited effects of pesticides on stream macroinvertebrates, biofilm nematodes, and algae in intensive agricultural landscapes in Sweden.</b> Water Research, 27, 115640.</p>	
<p><b>Location</b></p>	<p>Southern Sweden</p>
<p><b>Waterbody type</b></p>	<p>Streams and rivers</p>
<p><b>Landuse surrounding area</b></p>	<p>Agricultural</p>



<b>Sampling information</b>	Algae, diatoms and nematodes were brushed off cobbles, kick sampling for macroinvertebrates and timeseries grab samples for water chemistry.
<b>Chemicals measured</b>	Pesticides (n=90) and metals (n=16).
<b>Aquatic species</b>	Algae and benthic diatoms, benthic macroinvertebrates and nematodes in benthic biofilms
<b>Biological metrics</b>	<p><b>Algae:</b> composition and biovolume of benthic algal communities</p> <p><b>Macroinvertebrates:</b> ASPT (Average Score Per Taxon), DJ-index for eutrophication, taxon richness, and SPEAR<sub>pesticides</sub> index</p> <p><b>Nematodes:</b> abundance and richness (NemaSPEAR [%] index)</p>
<b>Toxicity data used for model</b>	<p><b>Algae:</b> green algae, diatom and cyanobacteria (metals only) growth inhibition EC50s from PPDB for pesticides, ECETOX and EAT databases for metals.</p> <p><b>Daphnia:</b> 48h immobilisation EC50s from PPDB for pesticides and von der Ohe et al 2005 and ECETOX for metals.</p> <p><b>Nematodes:</b> similar to daphnia data as above</p>
<b>Mixture model</b>	Concentration addition
<b>Comparison between predicted mixture effects and field observations</b>	<p><b>Algae and macroinvertebrates:</b> community composition observed in the field did not correlate with the pesticide <math>\Sigma</math>TUs.</p> <p><b>Nematodes:</b> field community composition (NemaSPEAR [%] index) significantly correlated with pesticide <math>\Sigma</math>TUs for daphnia in summer 2016.</p>
<b>Key biological findings</b>	<p><b>Algae and macroinvertebrates:</b> lack of correlation may be due to i) underestimated exposure, ii) low pesticide run off, iii) eutrophication counteracting pesticide toxicity. Nutrient enrichment was the major driver of observed effects in diatoms rather than pesticides <math>\Sigma</math>TU.</p> <p><b>Nematodes:</b> correlation of <math>\Sigma</math>TU and observed effects may have been due to higher pesticide exposure of nematodes in biofilms.</p>
<b>Other findings</b>	- For algae $\Sigma$ TU for one sample exceeded the European Uniform



	<p>Principles (UP). For invertebrates only 2% samples exceeded UP and over 50% indicated reference conditions. 12 pesticides accounted for &gt;50% of <math>\Sigma</math>TUs. For <math>\Sigma</math>TU<sub>algae</sub> terbuthylazine, metazachlor and picoxystrobin were quantitatively the most important (i.e., &gt;65%). For <math>\Sigma</math>TU<sub>daphnia</sub> picoxystrobin, mecoprop and pirimicarb contributed most to the predicted effect.</p> <ul style="list-style-type: none"> <li>- SPEAR<sub>pesticides</sub> indicated that more than half of sites were had bad or poor status. SPEAR<sub>pesticides</sub> was positively correlated with the other biological metrics for macroinvertebrates but not <math>\Sigma</math>TU<sub>daphnia</sub>.</li> </ul>
<p><b>Plots of key results</b> RDA plot of all biological metrics and toxic units of pesticides (<math>\Sigma</math> TU) and metals (m <math>\Sigma</math> TU) for algae (a) and Daphnia (D). Explanatory variables are shown in blue. “D” = Diatoms, “BMI” = Benthic macroinvertebrates, “Nema” = Nematodes, “_S” = Taxon richness and “Def” = Diatom deformities (%). Continuous lines indicate variables that contribute significantly to the variation in biological metrics (Monte Carlo permutations, p &lt; 0.05)</p>	

**Case study 5. Marine species biomarkers and chemical mixture exposure**

<p>Lima, I., Moreira, S. M., Rendón-Von Osten, J., Soares, A. M., &amp; Guilhermino, L. (2007). <b>Biochemical responses of the marine mussel <i>Mytilus galloprovincialis</i> to petrochemical environmental contamination along the North-western coast of Portugal.</b> Chemosphere, 66(7), 1230-1242.</p>	
<p><b>Location</b></p>	<p>Portugal, NW coast</p>
<p><b>Waterbody type</b></p>	<p>Marine, intertidal zone</p>
<p><b>Landuse surrounding area</b></p>	<p>Various (seaports, harbours and beaches)</p>
<p><b>Sampling information</b></p>	<p>5 sampling sites along coast. Mussels were handpicked in intertidal zone.</p>
<p><b>Chemicals measured</b></p>	<p>aliphatic hydrocarbons (AH), unresolved complex mixture (UCM) and PAHs</p>
<p><b>Aquatic species</b></p>	<p><i>Mytilus galloprovincialis</i></p>
<p><b>Biological metrics</b></p>	<p>Biomarkers (SOD, CAT, GPx, GR, tGSx, GSSG, GSH/GSSG, IDH, ODH, LPO)</p>

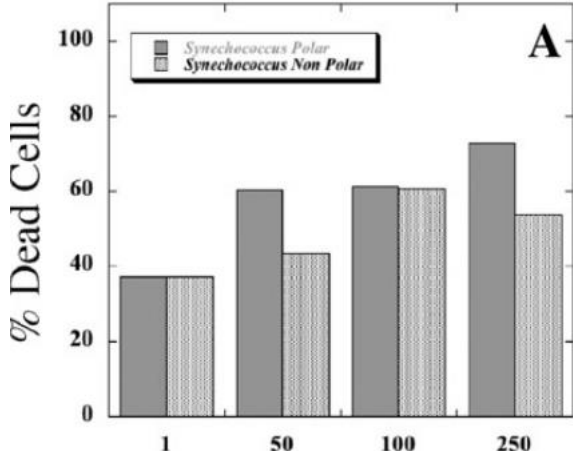


<b>Toxicity data used for model</b>	N/A
<b>Mixture model</b>	N/A
<b>Comparison between predicted mixture effects and field observations</b>	N/A
<b>Key biological findings</b>	Differences in the biomarker expression patterns between sampling sites were driven by unresolved complex mixture and PAH concentrations measured in mussel tissue as well as other abiotic factors like nitrate, ammonia, salinity, and pH.
<b>Plot of key results</b> Redundancy analysis (RDA) ordination diagram with sampling sites S1-S5 (dots), environmental parameters (thick arrows), and biochemical parameters (thin arrows)	<p>The RDA ordination diagram shows the relationship between sampling sites (S1-S5) and various parameters. Environmental parameters (thick arrows) include PAH, pH, PO<sub>4</sub>, NH<sub>4</sub>, NO<sub>2</sub>, NO<sub>3</sub>, and S. Biochemical parameters (thin arrows) include GPx, LPO, ODH, UCM, CAT, IDH, GSH/GSSG, GST, SOD, and AH. Sites S1, S3, and S4 are clustered in the lower-left quadrant, while S2, S4, and S5 are clustered in the upper-right quadrant. The x-axis ranges from -1 to 1, and the y-axis ranges from -1 to 1.</p>

**Case study 6. Marine phytoplankton and chemical mixture exposure**

Echeveste, P., Galbán-Malagón, C., Dachs, J., Berrojalbiz, N., Agustí, S. (2016). <b>Toxicity of natural mixtures of organic pollutants in temperate and polar marine phytoplankton.</b> Science of the Total Environment, 571, 34-41.	
<b>Location</b>	Mediterranean Sea, North-East Atlantic Ocean and Southern Ocean
<b>Waterbody type</b>	Oceans
<b>Landuse surrounding area</b>	N/A
<b>Sampling information</b>	Seawater samples collected and concentrated (above environmentally relevant concentrations) for experimental treatments of different organic pollutant concentrations
<b>Chemicals measured</b>	Organic pollutants (HCHs, HCBs, PCBs and PAHs)
<b>Aquatic species</b>	Phytoplankton
<b>Biological metrics</b>	Species abundance (via microscopy identification), total phytoplankton abundance (via chlorophyll a concentrations) and proportion of living versus dead cells in picophytoplankton communities



<b>Toxicity data used for model</b>	N/A															
<b>Mixture model</b>	N/A															
<b>Comparison between predicted mixture effects and field observations</b>	N/A															
<b>Key biological findings</b>	Toxic effects of POPs to marine phytoplankton at concentrations well above the field observations (i.e., environmentally relevant levels). A decline in phytoplankton populations and decreased growth was observed in experimental treatments relative to the control.															
<b>Plot of key results</b> Proportion of dead cells (relative to the control) of phytoplankton across the different concentration treatments of POPs.	 <table border="1"> <caption>Data for Plot of key results</caption> <thead> <tr> <th>Concentration</th> <th><i>Synechococcus Polar</i> (% Dead Cells)</th> <th><i>Synechococcus Non Polar</i> (% Dead Cells)</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>~35</td> <td>~35</td> </tr> <tr> <td>50</td> <td>60</td> <td>~42</td> </tr> <tr> <td>100</td> <td>60</td> <td>60</td> </tr> <tr> <td>250</td> <td>~72</td> <td>~53</td> </tr> </tbody> </table>	Concentration	<i>Synechococcus Polar</i> (% Dead Cells)	<i>Synechococcus Non Polar</i> (% Dead Cells)	1	~35	~35	50	60	~42	100	60	60	250	~72	~53
Concentration	<i>Synechococcus Polar</i> (% Dead Cells)	<i>Synechococcus Non Polar</i> (% Dead Cells)														
1	~35	~35														
50	60	~42														
100	60	60														
250	~72	~53														

**Case study 7. Relationships between mixture pressure and bioactivity biomarkers**

Blackwell, B. R., Ankley, G. T., Bradley, P. M., Houck, K. A., Makarov, S. S., Medvedev, A. V., ... & Villeneuve, D. L. (2019). <b>Potential toxicity of complex mixtures in surface waters from a nationwide survey of United States streams: Identifying in vitro bioactivities and causative chemicals.</b> Environmental science & technology, 53(2), 973-983.	
<b>Location</b>	US and Puerto Rico
<b>Waterbody type</b>	Streams
<b>Landuse surrounding area</b>	Range of land use including largely undeveloped, agricultural, and heavily urban developed watersheds
<b>Sampling information</b>	Surface water samples (n=38)
<b>Chemicals measured</b>	386 analytes (pesticides, pharmaceutical, nutrients, wastewater indicators, steroids and sterols, halogenated organics)
<b>Aquatic species</b>	N/A
<b>Biological metrics</b>	ToxCast High throughput screening (HTS) assays using HepG2 cells for bioactivity (69 biological cis_Factorial endpoints) + 8



	subsamples screened for 24 additional nuclear receptors (trans_Factorial endpoints)
<b>Toxicity data used for model</b>	High throughput screening (HTS) data for bioactivity from ToxCast program which screened 3600 compounds
<b>Mixture model</b>	Concentration Addition
<b>Comparison between predicted mixture effects and field observations</b>	<p>Bioactivity was detected for 11 endpoints in cis_factorial assays and &gt;3 endpoints in trans_factorial assays for <i>in vitro</i> receptor linked reported gene systems covering many key receptors known to be involved in the AOPs of different chemicals. Greatest activity was associated with the regulation of metabolism of xenobiotic or endogenous compounds, oxidative stress and different endocrine pathways. Effect causing the biological activation of 11 receptors accounts for over 95% of the positive activations by the collected samples (VDRE_CIS, PXRE_CIS, PXR_TRANS, PXR_TRANS, RxRb_TRANS, ERE_CIS, ER<math>\alpha</math>_TRANS, GR_TRANS, PPAR<math>\gamma</math>_TRANS, AhR_CIS, RORE_CIS), with the pregnane X receptor (PXR) and AhR-receptor activations the most commonly observed activation responses. When receptors were activated <i>in vitro</i>, observed responses could not be completely be accounted for by detected chemicals indicating that many chemical may contribute to receptor activation. Overall, prediction of bioactivity from individual measured chemical concentrations was not possible for the majority of endpoints. Only reasonable estimates could be made for estrogenic activity for very well known and mechanistically linked chemical ligands (estrone, 17<math>\beta</math>-estradiol, bisphenol A, and 4-nonylphenol). Other biological activation for critical effects pathways are varied with many chemical likely to contribute.</p>
<b>Key biological findings</b>	Several of the measured bioactivity endpoints are induced by well- defined ligands interacting with the pathway receptors. The analysis pipeline makes it possible to use these reporter assays to identify a number of biologically active



	<p>compounds in waters. All receptors can be mechanistically linked to adverse outcome pathways that have biological meaning apical effects.</p>
<p><b>Other findings</b></p>	<ul style="list-style-type: none"> <li>- Land Use Land Cover (LULC) effects on bioactivity: Significant correlation of bioactivity percent of watershed area classified as developed, low intensity, number of registered National Pollution Discharge Elimination System (NPDES) facilities, and Catchment-scale medium and high intensity development.</li> <li>- AhR and PXR activity was associated with low intensity, developed land cover and endocrine-related end points ER and glucocorticoid receptor was frequently linked to waste water discharges. Together, this suggests that anthropogenic inputs were the main drivers of the observed effects. However, no strong predictive relationship was found with any single LULC.</li> <li>- HTS assays showed lower sensitivity than some targeted transcriptional reporters assays, however, their results aligned.</li> </ul>
<p><b>Plots of key results</b></p> <p>An example radar plot of blank-normalized area under the curve (AUC) values of both cis_Factorial and trans_Factorial end points for the South Platte River, CO site. Assay end point names correspond to those in Table S2 in the paper and are grouped in colors by general function. The radar axis represents AUC values for each measured end point. The activity cutoff value of 1.5 is shown in bold, and activities at or above this value were identified as active.</p>	<p style="text-align: center;">Site: S. Platte R, CO</p> <p style="text-align: right;">Assay Classification</p> <ul style="list-style-type: none"> <li>Cell Stress</li> <li>Endocrine</li> <li>Growth/Differentiation</li> <li>Immune</li> <li>Lipid metabolism</li> <li>Metabolism</li> <li>Xenobiotic metabolism</li> </ul>

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