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Environmental risk assessment of combined effects in aquatic ecotoxicology: a discussion paper

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ABSTRACT

Environmental regulatory edicts within the EU, such as the regulatory framework for chemicals REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals), the Water Framework Directive (WFD), and the Marine Strategy Framework Directive (MSFD) focus mainly on toxicity assessment of individual chemicals although the effect of contaminant mixtures is a matter of increasing concern. This discussion paper provides an overview of the field of combined effects in aquatic ecotoxicology and addresses some of the major challenges related to assessment of combined effects in connection with environmental risk assessment (ERA) and regulation. Potentials and obstacles related to different experimental, modelling and predictive ERA approaches are described. On-going ERA guideline and manual developments in Europe aiming to incorporate combined effects of contaminants, the use of different experimental approaches for providing combined effect data, the involvement of biomarkers to characterize Mode of Action and toxicity pathways and efforts to identify relevant risk scenarios related to combined effects are discussed.

Key words: Environmental pollutants, combined effects, risk assessment, regulation

1. Introduction

Organisms in polluted environments are typically exposed to a complex mixture of chemical contaminants and the exposure may sometimes cause toxic effects even though the individual stressors are present at concentrations lower than the No Observable Effect Concentration (NOEC) (Brian et al., 2007; Kortenkamp, 2008; Silva et al., 2002). This phenomenon is known as combined effects, mixture toxicity, joint toxicity or cocktail effects. Because the assessment of chemical toxicity normally is done substance by substance, neglecting potential mixture effects, it is possible that adverse effects of environmental pollutant mixtures are underestimated. Contaminants with similar or different Mode of Action (MoA) can influence each other's toxicity; resulting in an almost unlimited number of possible additive, synergistic or antagonistic combinations. The term MoA can be defined as the series of key processes that begins with the interaction of a chemical contaminant with a target (e.g. receptor) site and proceeds through operational and anatomical changes in an organism that result in sublethal or lethal effects (USEPA, 2000). Due to the large number of potential chemical contaminants and the great complexity of natural systems it is not feasible to perform (eco)toxicity tests for each potential mixture. In addition, non-chemical factors may also act as stressors and add to the complexity of multiple stressor situations (Figure 1). Therefore, a simplified and robust approach to assess the ecotoxicity of chemical mixtures is needed for use in environmental risk assessment (ERA) and in regulatory toxicology. ERA is defined as procedures by which the likely or actual adverse effects of pollutants and other anthropogenic activities on ecosystems and their components are estimated with a known degree of certainty using scientific methodologies (Depledge and Fossi, 1994). An ERA framework normally includes a certain set of tiered modules as shown below (Figure 2) and provides a tool for evaluation and management of environmental pollution. The aspects of combined effects have not yet been implemented in ERA in a standardised manner, nor has the combined effect issue become an integrated part of chemical regulation edicts (Kortenkamp et al., 2009). However, an active process aimed for meeting these limitations has been going on for some time.

In this paper, the status in the field of combined effects is discussed, with emphasis on issues related to aquatic environments. Although research on combined effects has gained impetus recently many major gaps of knowledge remain; such as: which environmental

pollutants (classes and specific structures) are likely to contribute most significantly to combined effects? What are the predominant cause-effect relationships and MoAs involved? Which non-chemical factors are relevant? In which phyla does combined effects occur at environmentally realistic conditions and how pronounced are species-differences in susceptibility? And how can issues of combined effects become implemented in ERA and environmental regulation? The discussion will be oriented around the following set of ecotoxicological problem formulations:

1) Which biological species/organization level do we aim to protect (individual, population community, ecosystem keystone species)?
2) Which endpoints/effects do we consider being relevant (e.g. the regulatory endpoints)?
3) Which compounds do we expect to encounter (from monitoring data)?
4) Which compounds are likely to cause effects (based on persistence, bioaccumulation/biomagnification, and toxicity (PBT) criteria)?
5) Which assemblies of compounds are likely to cause combined effects (given possibly relevant MoA and effect endpoints)?
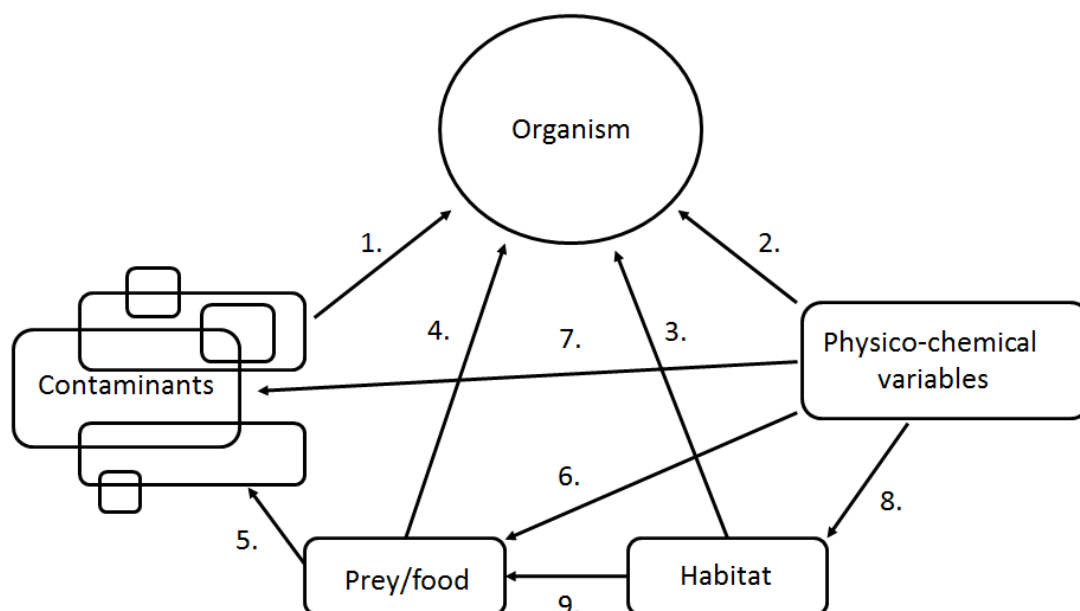
Figure 1: Multiple factors which may affect the organism as stressors. 1: Exposure and effect of contaminants (possible outcomes being additivity/synergism/antagonism). 2: Physicochemical variables (e.g. climatic conditions). 3: Habitat changes. 4: Availability, type and nutritional value of food. 5: The type of food influence type and magnitude of contaminant exposure. 6: physical variables influence availability of food (e.g. abundance of prey species). 7: Changes in environmental variables influence contaminant

bioavailability (e.g. by transport/advection, diffusion, adsorption etc.). 8: Physicochemical variables also affect the habitat of the organism. 9: The habitat of the organism is also the habitat of its prey organism, thus influencing on type and availability of food.
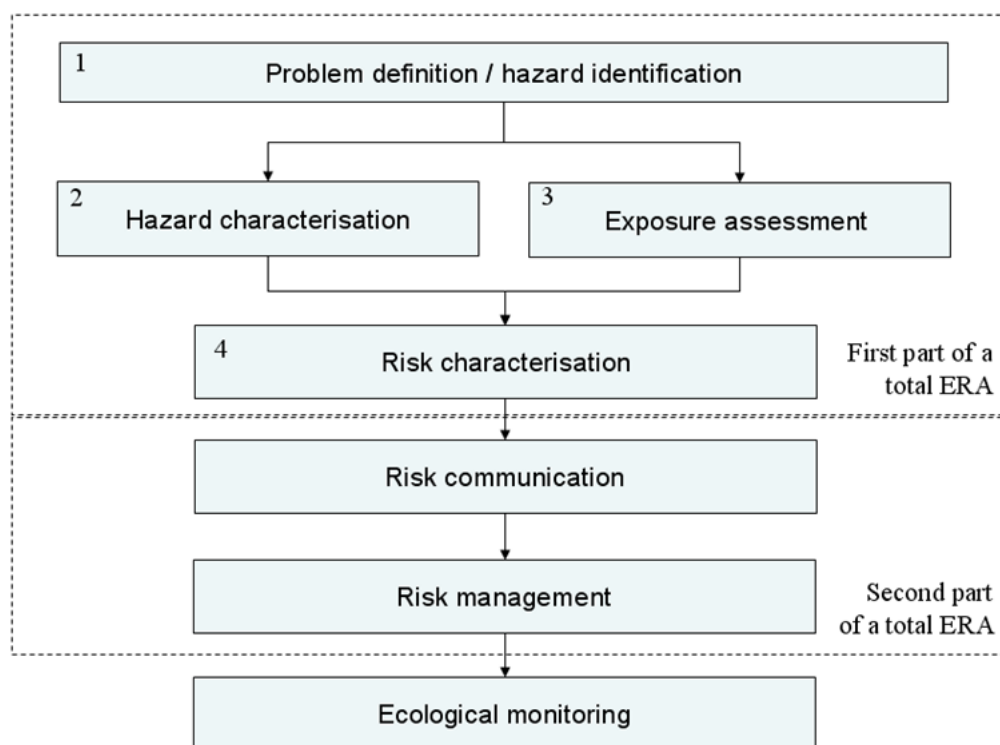

Figure 2: Conceptual overview of the main components of an Environmental Risk Assessment (ERA) framework. Such frameworks are widely used to organize the processes of assessment and management of chemical pollution.

2. Anthropogenic contaminant stresses relevant to combined effects

Pesticides have received much attention as possible combined toxicity stressors in different aquatic environments (Relyea, 2009; Rodney et al., 2013; Verbruggen and Van den Brink, 2010). The term *pesticide* refers to any (toxic) substance used for the purpose of combating a pest organism. Some pesticides (in particular the organohalogens) are highly persistent in the environment and according to the Stockholm convention on POPs are as many as 9 of the 12 most environmentally hazardous organic chemicals pesticides. Certain animal classes, such as the amphibians, are thought to be particularly sensitive to the combined toxicity of pesticide mixtures, e.g. Hayes et al. (2006). It is a concern that the significant decline recorded in amphibian populations in many agriculturally dominated

regions around the world is, at least partly, caused by the adverse effect of pesticide mixtures.

The so-called persistent organic pollutants (POPs) including polychlorinated biphenyls (PCBs) and polybrominated flame retardants and many other substance classes, constitute a diverse class that is considered as relevant in connection with mixture toxicity phenomena, especially in ecological top-predators such as seals, cetaceans, otters and birds of prey, as well as in humans. In some populations of top-predatory animals, significant reductions of the total reproductive outcome have been found to coincide with increased long-term exposure to highly biomagnified levels of POPs. Among the best known case-studies is the four-decade long investigation in the Baltic grey seal population, which during the 1960s and 1970s became more and more diminished. The decline was apparently a result of lowered reproductive success since many females had lost the ability to give birth because of occlusions, obstructions and tumors in the uterus. These pathological changes in the seal uteri were shown to correlate with the concentration of a mixture of organochlorine contaminants (Helle et al., 1976). High levels of organochlorines did not only correlate with pathological uterus lesions, but also with a larger disease complex including lesions on skin, claws, intestines, kidneys, the adrenal gland and skeleton (Bergman and Olsson, 1985; Mortensen et al., 1992). It was hypothesized that methyl sulfone metabolites of DDE or PCBs (Bakke et al., 1982) were responsible for the reproduction problems of the Baltic seals, due to their disposition to accumulate in endocrine tissue in the adrenal cortex and causing adrenocortical hyperplasia (Bergman and Olsson, 1985). Interestingly, a significant improvement of the reproductive status of the seal population was seen during the period 1990-2010, coinciding with a markedly decrease in the PCB levels (almost 90% reduction) and DDT levels (more than 90% reduction) measured in seal lipids (Roos et al., 2012). The similar trend of reduced POP levels and improved reproductive outcome was also observed in populations of otters and eagles within the Baltic area (ibid.). Studies in Canadian beluga whales (*Deiphinapterus leucas*), have suggested that immunosuppression is a key MoA for mixtures of POPs, leading ultimately to reproduction failure and population recruitment depression as a long-term effect (Deguise et al., 1995). However, the relationship between the long-term exposure to complex mixtures of POPs and development of reproduction impairment is most likely a complex and multistep effect process.

Aryl hydrocarbon receptor (AhR) agonists and antagonists constitute the groups of chemicals which have an ability for interacting with AhR which is an important transcription factor in connection with regulation of detoxification enzymes and many other proteins (Denison and Nagy, 2003; Hankinson, 1995). For example, cytochrome P450 1A (CYP1A), the major phase I detoxification enzyme in fish (Goksøyr and Førlin, 1992), is strongly up-regulated after exposure to potent AhR agonists including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), numerous carcinogenic polycyclic aromatic hydrocarbons (PAHs) and coplanar PCBs. CYP1A induction is therefore used as a biomarker for the presence of AhR agonists in an exposure situation. In cases when multiple Ah receptor agonists occur together, both additive and synergistic activity may happen (Billiard et al., 2006; Chaloupka et al., 1993; Kortenkamp, 2007), but also antagonistic mixture effects can occur, e.g. (Besselink et al., 1998). For example, suppressive effects of the CYP1A induction response has been observed when fish are exposed to toxic trace metals (Cd and Cu) and AhR agonists at the same time (Benedetti et al., 2009; Benedetti et al., 2007; Beyer et al., 1997; Sandvik et al., 1997).

Endocrine Disrupting Chemicals (EDCs) are highly relevant stressors in connection with combined effects (Kortenkamp, 2007). EDCs are hormonally active substances that can act as agonists or antagonists to hormone receptors or in other and more indirect ways perturb endocrine control systems (Colborn et al., 1993; Vos et al., 2000). EDCs are of special concern since they affect essential biological competences such as growth, development and reproduction, and because they can be active at extremely low concentrations (Rotchell and Ostrander, 2003). In connection with mixture effects, the possible endocrine disruptive effects on biological processes regulated by steroid hormones (e.g. estrogenic and anti-androgenic actions) (Rajapakse et al., 2002), and thyroid hormones (Crofton et al., 2005; Flippin et al., 2009; Kortenkamp, 2007) have been much in focus. Most mixture effect studies of EDCs have addressed estrogen receptor (ER) agonist issues. For example, the study in freshwater fish by Brian et al. (2005) showed that a combined response to a multicomponent mixture of ER agonists can be predicted by using a concentration addition (CA) approach and with vitellogenin (vtg) induction in male fathead minnows (*Pimephales promelas*) as a biomarker for the estrogenic effect. They showed that the effect of the chemical mixture detected *in vivo* were highly comparable with those predicted by a bio-mathematical model and *in vitro* studies using isolated

rainbow trout (*Oncorhynchus mykiss*) liver cells (hepatocytes) and transgenic zebrafish embryos (Petersen and Tollefsen, 2011). Similar findings have been presented by Thorpe et al. (2003; 2001) who studied the combined effect of ER agonists on the vtg production in juvenile rainbow trout.

Mixtures of toxic elements, such as Hg, Pb, Cd, or metal-associated toxicants such as methylmercury, tributyltin, alkyllead, and others, may occur in polluted aquatic systems and may be expected to cause combined toxicity (Bryan and Langston, 1992; Kadokami et al., 2013; McCready et al., 2006; Norwood et al., 2003; Rainbow, 1995). In some marine top-predatory fish, such as Atlantic and Mediterranean swordfish (*Xiphias gladius*), high concentrations of Hg, Pb and Cd are found and it is a concern that this chronic contamination through combined toxicity effects may have affected reproduction performance and contributed to an observed population decrease (Damiano et al., 2011). Combined toxicity of metals can also occur in organisms at lower trophic levels. Fukunaga et al. (2011) found that metal mixtures (e.g. copper and zinc) gave an additive toxicity that influenced the tendency of recolonization of defaunated estuarine sediments. Additive effects were detected for the general species richness, for the mean log abundances of several polychaete species and for the multivariate response of the community as a whole. Fukunaga et al. (2011) suggest that characterizing the combined effect potentials of heavy metal mixtures to sediment infaunal communities is essential in order to build better predictive models for environmental risk assessments of metal pollution situations.

Long-living animal species at the top of the food chain may be under particular risk for combined effects due to the biomagnification of toxic substances along the food chain. Mixtures of persistent organohalogen contaminants and some inorganic toxicants are found in highly biomagnified levels in top-level predators from different animal classes, and even in remote Arctic regions (Barrie et al., 1992; Borgå et al., 2004; Braune et al., 1999; de Wit et al., 2006; Muir et al., 1999). The Arctic contamination phenomenon is thought to result from the long-range transport of POPs from lower latitude to high latitude regions and to biomagnification of these substances along Arctic food chains (Bard, 1999; Hung et al., 2010; Wania and Mackay, 1993). Studies in polar bears from Svalbard show that in particular male bears accumulate high levels of higher chlorinated PCBs (Bernhoft et al., 1997; Norheim et al., 1992) and that the levels of PCBs correlate with markers of reproductive success as well as various biochemical and physiological markers, including

levels of thyroid hormones, retinol (vitamin A), immunoglobulin G, and enzymatic detoxification activity (Skaare et al., 2000). Other Arctic top predators such as the glaucous gull (*Larus hyperboreus*), polar (Arctic) fox (*Vulpes lagopus*), and Arctic char (*Salvelinus alpinus*) have been subjected to similar research studies (Bustnes et al., 2003). Letcher et al. (2010) summarizes recent studies on biological effects in Arctic wildlife in relation to exposure to complex organohalogen mixtures, and attempts to assess *in-vivo* concentration data in the context of possible threshold levels of effects to evaluate the risks. The review concludes that apart from East Greenland and Svalbard polar bear populations and Svalbard glaucous gulls, there is still little confirmatory evidence of contaminant induced stress in Arctic populations (Letcher et al., 2010). It is also emphasized that the influence by other anthropogenic and natural stressors/factors renders a picture so complex that the identification of a direct link between contaminant exposure and long-term biological effects in Arctic populations becomes extremely difficult. They warn that field studies that address relationships between contaminant exposure and putative effects in Arctic wildlife will typically be of correlative nature and will therefore not provide true cause-effect documentation, although they are important in a weight of evidence (WoE) approach (Weed, 2005).

3. Top-down evaluation of chemical mixture effects

Since the late 1980's, several top-down oriented test strategies have been developed that use biological responses to direct the identification of causal agents in chemical mixtures. The most relevant of these are Effect-Directed Analysis (EDA) and Toxicity Identification & Evaluation (TIE). The EDA procedure includes a combined use of chemical fractionation, sequential bioassay and subsequent chemical analyses and builds on the assumption that toxicity can be assessed for separated classes of chemicals or for matrices deprived of specific classes of chemicals (Brack et al., 2007; Brack and Schirmer, 2003; Burgess et al., 2013; Hecker and Hollert, 2009; Samoiloff et al., 1983). The TIE procedures were developed by US EPA, as one of the first standardised EDA procedures, and mainly used for identification and evaluation of contaminants in aqueous samples (Brack et al., 2008; de Vlaming et al., 2000; Mount and Anderson-Carnahan, 1988). Basically, the concept in TIE is to remove groups of compounds with certain properties (e.g. organics, metals, ionic and non-ionic compounds) from a test matrix until the toxicity

of the sample disappears. Then, suspected chemicals are identified by analytical chemistry, and lastly their toxicity is confirmed by means of the same bioassay as used in the initial toxicity characterization phase (Hecker and Giesy, 2011). The TIE procedures have later been further developed to also enable toxicity evaluations of sediments matrices. As discussed by Burgess et al. (2013), EDA and TIE approaches have fundamental differences that make them distinct techniques. EDA uses primarily mechanism-specific *in vitro* bioassay endpoints whereas TIE methods typically determine active toxicants to whole-organism endpoints. In EDA, the fractionation and chemical analyses performed to identify the causes of toxicity may often compromise contaminant bioavailability; whereas in TIE, toxicant bioavailability is maintained and is considered critical for identifying the causes of toxicity. However, both EDA and TIE approaches have limitations with regard to assessing the nature and magnitude of combined toxicity, such as additivity (see *Bottom-up evaluation (prediction) of chemical mixtures* for details), synergism (i.e. larger effect than expected on the basis of additivity predictions) and antagonism (i.e. smaller effect than expected on the basis of additivity predictions). In connection with a top-down study approach, the involvement of ecotoxicity tests and biomarkers might represent a means for identifying the major targets for toxicity, for quantifying the adverse effect of concern and for defining "key events" along the sequence of biological responses leading to certain toxicological endpoints. Targeted effect studies can be conducted to identify different suites of cellular, subcellular and biomolecular biomarkers that are responsive to individual contaminants and various mixtures in question (van der Oost et al., 2003; Walker, 1998). In recent years, the development of high resolution mass spectrometry techniques combined with extensive compound libraries has greatly increased the feasibility of identifying substances within complex mixtures. Accurate mass measurements over a full spectrum make it possible to screen for a large number of organic contaminants at low levels. An advantage of this technique is that the data remain available for subsequent analysis, and a retrospective analysis can be performed at a later stage if needed (Hernández et al., 2011; Hernández et al., 2012). In the future, the combination of more advanced chemical analytical tools and more detailed knowledge about biological effects can be expected to increase the potential for a successful top-down approach.

4. Bottom-up evaluation (prediction) of chemical mixture effects

Toxicants contributing to combined effect are thought to exert their effect along two major avenues, namely by concentration addition (CA, also called dose addition or Loewe additivity), or by independent action (IA, also called response additivity or Bliss independence) (Altenburger et al., 1996; Altenburger et al., 2003; Goldoni and Johansson, 2007; Greco et al., 1995; Suhnel, 1998). CA occurs when two or more chemicals with similar MoA affect the same target of toxic action (endpoint), whereas IA occurs when two or more chemicals affect the same endpoint but through dissimilar MoAs (Figure 3). The concept of CA was originally introduced by Loewe and Muischnek (1926) and Loewe (1927) and can be mathematically explained by the equation (1):

$$ECx_{(Mix)} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1} \tag{1}$$

Where $ECx_{(mix)}$ is the predicted total concentration of the mixture that induces x% effect, p_i is the relative fraction of component i in the mixture and ECx_i is the concentration of substance i provoking a certain effect x when applied alone.

The concept of IA was first applied to biological data by Bliss (1939) and can be mathematically explained by the equation (2):

$$E_{Mix} = 1 - ((1 - E_1) * (1 - E_2) * \ldots (1 - E_n)) = 1 - \prod_{i=1}^{n}(1 - Ei) \tag{2}$$

Where E_{Mix} is the effect of the mixture of n compounds and E_i is the effect of substance i when applied singly.

The CA and IA models can be used to make predictions of combined effects on several endpoints, including acute toxicity (Faust et al., 2003; Tollefsen et al., 2012) and endocrine disrupting effects (Brian et al., 2005; Petersen and Tollefsen, 2011, 2012; Thorpe et al., 2003; Thorpe et al., 2001). Deviations of experimental data from the model estimates are commonly identified as synergistic or antagonistic effects, with ample reports of additivity, synergism and antagonism occurring in literature, see (Altenburger et al., 2003; Belden et al., 2007) for reviews. There is currently no common approach for how to decide whether a deviation from the prediction is large enough to accurately identify synergistic or

antagonistic effects. Thus several methods have been employed to compare observed mixture effect data with the additivity expectations, whereof some are mentioned below. The bootstrap method used by Brian et al. (2005) enables 95% confidence interval (CI) to be derived for the mean predicted effect, and observed data falling within the 95% CI of the predicted values can thus be said to be additive. The model deviation ratio (MDR) used by Belden et al. (2007) is calculated by dividing the predicted effect concentration by the observed effect concentration and the results are often said to be additive if the MDR is within a factor of 2. However, for effect curves with a steep slope, this approach might not be optimal as the observed and estimated effect at a certain concentration can be substantially different. This is of highest concern when the observed data have a stronger effect than the additivity expectations, indicating that the model underestimates the actual risk. Some studies have derived 95% CI for the regression model fitted to the observed data and identify additive effects when the additivity expectations are within these limits (Petersen and Tollefsen, 2011, 2012). The 95% CI is highly dependent on the variation within the dataset, and a dataset with a large variation can have a 95% CI that overlaps with the prediction models even when the effect concentrations (ECx) of the observed and predicted data can be substantially different. In general, there appears to be a need for a standardized approach for evaluating observed effects by use of CA and/or IA in order to standardize the criteria for additive effects.

Only a few studies have shown successful predictions of combined effects of independently acting chemicals in aquatic test system (Faust et al., 2003). To date, no mammalian studies have shown combined effects predicted by IA models. Although the concept of IA is important to consider in certain cases, often the CA model can be used even if some chemicals are known to have independent MoAs. This is especially the case when the mixtures tested include a large number of compounds. Increasing number of compounds will increase the possibility of interactions due to the complex pathway system (e.g. pathway cross-talks) and inter- and intracellular signalling in/between the target cells, decreasing the possibility of strictly independent acting chemicals. Two review studies on applicability of CA and IA models state that in most cases the effect of the investigated mixtures is well predicted by these models (Belden et al., 2007; Cedergreen et al., 2008). Approximately half of 158 evaluated data sets for the combined toxicity of binary mixtures of primarily pharmaceuticals and pesticides in small scale test systems (*Vibrio fischeri,*

activated sludge microorganisms, *Daphnia magna, Pseudekirchneriella subcapitata, Lemna minor, Tripleurospermum inodorum* or *Stellariamedia*) could be adequately predicted by either CA or IA (Cedergreen et al., 2008). Synergistic interactions were observed for only 6% of the mixtures (Ibid.). In the review by Belden et al. (2007) as much as 88% of the investigated combined toxicity studies of pesticide mixtures to aquatic life was successfully predicted by the CA model, whereas only approximately 10% of the tested mixtures were identified to cause interactions that significantly affected toxicity.

In general, CA or IA models have limited applicability with real field data. CA and IA model tools are designed for making predictions of combined effects (from theoretical viewpoints) and for making study-hypotheses on combined effects that subsequently can be tested/validated with the use of empirical effect data from laboratory controlled exposure studies. However, the use of mesocosms for combined toxicity studies has in several studies been demonstrated as a feasible and more field-realistic approach, e.g. (Knauert et al., 2008; Sura et al., 2012).

5. Use of toxicogenomics in combined toxicity studies

Environmental stressors will usually have more than one MoA and may display interactions with multiple targets along an adverse outcome pathway (AOP) that comprise interaction with a molecular target, modulation of key events associated with the stress response and ending with the adverse effect of concern (Figure 3). When the CA or IA models have limitations to predict combined toxicity based on a known endpoint, more in-depth knowledge on the toxicological mechanisms can be provided by non-biased and discovery-driven approaches such as that provided by toxicogenomics (OMICS). A conceptual framework for combined toxicity studies making use of OMICS approaches to support AOP development was recently proposed by Altenburger et al. (2012). The rapid development of toxicogenomics and associated high-throughput methods have greatly facilitated the characterization of both key molecular events and complex sequential key events caused by stressors based on the measurements of genomic modifications (epigenetics), transcription of genetic information to mRNA (transcriptomics), translation of information from mRNA to protein synthesis (proteomics) and metabolic activities and related products (metabolomics). The ultimate purposes of using OMICS oriented approaches in mixture toxicity studies are: to build a more complete overview of stress-

response profiles (e.g. toxicity pathways) for both single stressor and the mixtures; to identify key MoAs and mechanisms of action (MOAs) for categorization of stressors in the mixture design; to mechanistically understand the potential interactions of stressors; and more importantly, to shed light on the selection of robust biomarkers for mixture prediction models in ERA. In addition, the OMICS tools are suitable to study the effects of stressors at low concentration/dose (e.g. at NOEC level), as the molecular endpoints are relatively more sensitive than conventional toxicological endpoint such as survival, growth and reproduction, although the OMICS data may not indicate a toxicity condition as some cellular biomarkers can do. It should be noted that the molecular responses at very low exposure level or short exposure durations may not necessarily represent adverse outcomes at the physiological level, but may provide useful information on the stress-induced signal transductions and the defence system to maintain homeostasis in an organism (Song et al., 2012). The use of OMICS in studies of mixture toxicity has increased in recent years, but will still need development to accommodate the needs within combined toxicity assessment (Altenburger et al., 2012). Successful implementation of OMICS data into ERA may require supporting data from other components of an extended AOP, such as uptake, bioaccumulation (internal concentration) and apical effects (biomarker response and phenotypic anchoring).
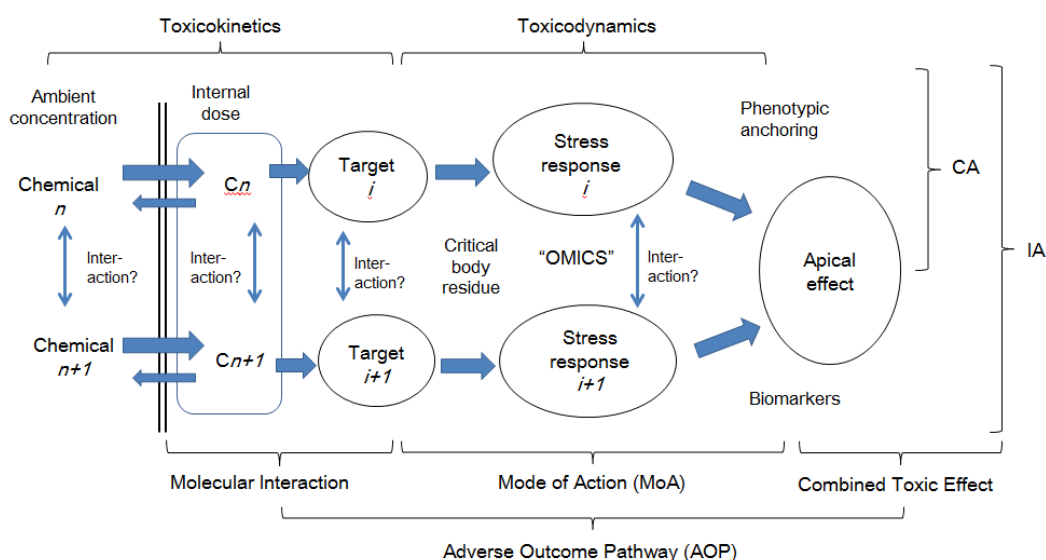

Figure 3: A conceptual framework of studying adverse outcome pathway (AOP) in a mixture design using toxicogenomic ("OMICS") approaches (modified from Altenburger et al., (2012)). CA: Concentration addition; IA: Independent action.

6. *In vitro* versus *in vivo* testing in mixture effect studies

For testing of mixture effects, *in vitro* studies offer an advantage over *in vivo* studies due to their high throughput and possibility to investigate specific MoAs. However, *in vivo* studies are in most cases considered more environmentally relevant than *in vitro* studies, as they also account for the complex whole organism feedback systems involved in regulation of organismal responses to environmental stress. Studies have shown that a number of genes are affected *in vivo* but not *in vitro*, e.g. for genes regulated by feedback system (Hultman et al., 2012). However, *in vitro* and *in vivo* responses can also be quite similar when looking at specific biomarkers, i.e. induction of vtg (ibid.). Detailed knowledge about the toxicity pathway involved thus opens for development and use of *in-vitro* based medium- and high-throughput cellular toxicity assays, e.g. (Petersen and Tollefsen, 2011, 2012), for studies of combined effects of contaminant mixtures (which typically involve a huge number of test combinations). In aquatic ecotoxicology, the use of *in vitro* fish cells and fish embryos have been proposed as an alternative to the use of (adult) *in vivo* tests (Castano et al., 2003; Embry et al., 2010; Lammer et al., 2009). Developments such as the recent acceptance (April 2013) of the fish embryo test by the Working Group of National Coordinators of the Test Guidelines Programme (WNT) for toxicity testing, provide promises for larger implementation of alternative test methods also in combined toxicity assessment and regulatory applications. For one of the most studied group of test compounds, the estrogen receptor agonists, there appears to be a good conformity in combined toxicity assessment in *in vitro* and *in vivo* bioassays. Both experimental approaches have identified CA as being most applicable to combined effect assessment of complex chemical mixtures (Brian et al., 2005; Petersen and Tollefsen, 2011; Thorpe et al., 2003; Thorpe et al., 2001).

7. Non-chemical factors in combined effects

Multiple stressor situations are often characterised by combinations of chemical and non-chemical stressors (e.g. Figure 1). Relevant non-chemical stressors may include physical factors (e.g. ionizing radiation, temperature stress, UV-irradiation), biotic stress (e.g. parasite, bacteria, virus infections) but also factors related to alterations of habitat (e.g. habitat loss, food shortage). A major non-chemical stressor is the prospective changes in climate conditions, especially in the Arctic region, where these changes are likely to be

of higher magnitude than the global mean (IPCC, 2001). In addition to posing stress themselves, changes in geophysical parameters induced by climate change may significantly change the environmental abundance of organisms as well as their body burdens of contaminants, e.g. Borgå et al. (2010). A warmer climate and more acidic and eutrophic oceans will potentially contribute to increased combined stress, leaving organisms more sensitive to even slight perturbations caused by contaminant chemicals, i.e. if the organisms are pushed to the limits of their physiological tolerance range (Hooper et al., 2013; Huntington, 2009; Kallenborn et al., 2011). Significant changes in food-web structure (e.g. induced by climate change) may pose several types of stress to organisms due to alterations in the nutritional value of their diets. Furthermore, these large scale perturbations may affect the food-web transfer of contaminants; for example it has been shown that changes in the feeding habits of polar bears from western Hudson Bay have resulted in increases in the tissue concentrations of POPs (McKinney et al., 2009). Increasing trends in dissolved organic carbon (DOC) have been shown in surface waters in boreal areas of North America and Europe, and is most likely a response to the decline in the sulphate content of atmospheric deposition (De Wit et al., 2007; Monteith et al., 2007). The DOC is derived from soil organic material and may act as a carrier for organic pollutants (Ding and Wu, 1997). Thus, an increase in DOC could contribute to the increased transport of old contaminants sorbed to dissolved humic substances and causing a wash-out of contaminants to marine areas/estuaries (Ruus et al., 2010). Plastic materials are additional global anthropogenic discharges that may contaminate the environment as persistent particulate debris, and which may affect remote marine regions (Barnes et al., 2010; Bergmann and Klages, 2012). It is known that plastic waste particles are ingested by many species and the presence of plastics is thought to contribute significantly to situations of combined stress in multiple marine species, including sea birds, turtles and mammals (Andrady, 2011; Cole et al., 2011; Hidalgo-Ruz et al., 2012; Moore, 2008; Wright et al., 2013).

8. Environmental risk assessment of combined effects

Possible strategies for improvement of ERA of chemical mixtures have been proposed by several research groups, e.g. (Backhaus and Faust, 2012; De Zwart and Posthuma, 2005), as well as by international bodies that address regulation and legislation of

chemicals, e.g. the biocides technical meetings under the European Commission (2012). In general, there is consensus concerning the need for developing a tiered approach for chemical mixtures in ERA, e.g. by adopting a primary screening step and a subsequent in-depth testing process. One possibility is to utilise a two-tiered process. Tier 1 may include the identification of interaction relevant chemicals in specific study-matrices through high-throughput toxicity screening and/or bioactivity profiling methods; and subsequently, through Tier 2, in which the more detailed interaction influence of relevant contaminant combinations on specified effect endpoints in selected model organisms is determined at various exposure doses/concentrations.

According to the European regulatory framework for chemicals (REACH) standardized toxicity tests using organisms from major trophic levels (primary producers, primary and secondary consumers) should be used for assessing the ecotoxicity hazard for (individual) chemicals. Subsequently, this information is used to estimate PNEC (Predicted No Effect Concentration) values as estimates of the putative effects that each contaminant might have in specific ecosystem situations. According to chapter R.10 in the guidance document for implementation   of   REACH (http://echa.europa.eu/documents/10162/13632/information_requirements_r10_en.pdf), PNECs should be derived from the most sensitive effect data set and by applying an appropriate assessment factor (AF) to compensate for the uncertainty that descends from intra- and inter-laboratory variation, biological variance, and extrapolation from laboratory to field situations and short-term to long-term effect scenarios (ECHA, 2008). For chemicals that are imported to EU in quantities exceeding 10 tons per year an AF value of 1000 must be used to calculate PNECs for aquatic environments in cases when only "the base set" toxicity data (i.e. short-term toxicity data for algae, crustaceans and fish) are available. The quotient of the predicted environmental concentration (PEC) of a chemical and its toxicity potential given by the PNEC value gives the so-called PEC/PNEC ratio (the Risk Quotient, RQ) which is widely used as a standardised measure of risk in ERA procedures (van der Oost et al., 2003). The recent technical workshop in the EC biocides group (2012) expressed that ERA of chemical mixtures is realistically achievable based on certain default assumptions and a well-defined tiered assessment scheme, consisting of the three major tiers (I) PEC/PNEC summation, (II) Toxic Unit Summation and (III) mixture testing. The meeting also emphasised that the quality of a mixture toxicity assessment is

depending on the adequate identification of relevant chemical components within the mixture of concern. The principle of using chemical data in biological samples for assessment of mixture toxicity has been discussed by SETAC in terms of a Tissue Residue (TR) Approach (Dyer et al., 2010). From that discussion, a framework was suggested that integrates TR data and mixture toxicity information in a 3-tier approach, in which Tier I uses CA to estimate the mixture toxicity regardless of MoA of contaminant components, whereas Tier II is a mixed model that employs CA and IA to estimate mixture toxicity, and Tier III provides an integration of the TR data with a "multi-substance Potentially Affected Fraction" (ms-PAF) method in order to derive TR levels which are protective of a selected percentage of organism species within the aquatic community of concern (e.g., hazardous concentration for 5% of the species).

Another interesting approach based on species sensitivity distributions (SSDs) could be possible in cases where a considerable amount of ecotoxicological data is available for all chemical components in the mixture. A SSD quantifies the fraction of species potentially affected in contaminated environmental compartments using sensitivity data of several test species (Aldenberg and Jaworska, 2000; Forbes and Calow, 2002; Wheeler et al., 2002). In cases when much effect information is available also the use of a detailed toxicokinetic and/or toxicodynamic modelling approach can be feasible. However, REACH requests only a basic set of data for most compounds, which normally is considered as insufficient for SSDs estimations or for modelling approaches.

Backhaus and Faust (Backhaus and Faust, 2012) recently presented a two-tiered outline for a predictive environmental risk assessment of chemical mixtures with effect assessments based on a CA approach as the first tier and considerations of IA effects as the second tier (Figure 4). The main concept of this approach is to make use of available effect data (PNEC, NOEC, EC_{50}, etc) and predicted environmental concentrations (PECs) to calculate RQs. Two different approaches to integrate the concept of CA in the ERA calculations of RQ were proposed. The risk quotients could be calculated based on the sum of toxic units (RQ_{STU}) or by the sum of PEC/PNEC ratios ($RQ_{PEC/PNEC}$), as summing up PEC/PNEC ratios might serve as a justifiable CA-approximation if only base-set data are available. $RQ_{PEC/PNEC}$ provides the more conservative approach, is often easier to apply but might violate the assumption of a common biological endpoint which is the default in the CA and IA prediction models. It is suggested that if $RQ_{PEC/PNEC}$ is above 1, calculation of

RQ$_{STU}$ can be the next step. Consideration of IA should be made if the RQ$_{STU}$ is above threshold.

The pros and cons in using a WoE approach in mixture effect studies have been addressed by Adams et al. (2005), Chapman (2007), Dagnino et al. (2008) and Benedetti et al. (2012). In this connection, a WoE approach can be defined as a quantitative method for combining various evidences in support of a hypothesis (Weed, 2005). Due to the great complexity and variability of marine ecosystems, multiple lines of evidence will normally be required to establish relationships between stressors and effects in biota. Adams et al. (2005) suggested the development of a WoE approach which can be applied in a sequential manner by (1) characterizing the study system which involves determining if target biota are impaired, assessment of food and habitat availability, and measuring contaminant levels in the environment, (2) assessing direct effects of contaminant exposure on target biota using biomarkers and assessing indirect effects of exposure using suites of bioindicators, and (3) applying standard causal criteria based on epidemiological principles and diagnostic health profiling techniques to assess potential causes of stress. Using the concept of WoE in connection with combined toxicity evaluations is in line with European directives which require member states to evaluate the ecological status through involvement of multiple quality indicators. However, the concept and definition of WoE have yet not been described in a standardised way and this lack of consensus hampers the broader use of WoE in connection with risk assessment, regulatory toxicology and mixture effect assessments.

Problem and scenario definition

Potentials for synergism or antagonism? — yes → Specific consideration

no

QSAR data

Experimental data

Data compilation for all individual components and organismic groups considered

PNECs available for all components? — no

yes

Calculation of the sum of PEC/PNEC values ($RQ_{PEC/PNEC}$)

$RQ_{PEC/PNEC} >$ threshold? — no

yes

Calculation of the sum of toxic units for each group of organisms (RQ_{STU})

Selection of most sensitive group, application of AF

$RQ_{STU} >$ threshold? — no → No significant risk

yes

Mixture components at least partly dissimilarly acting? — no

yes

Relevant differences CA and IA possible? — no

yes

Consideration of IA (additional studies)

Predicted toxicity > threshold? — yes → Possible risk (further studies, risk management)

no

No significant risk

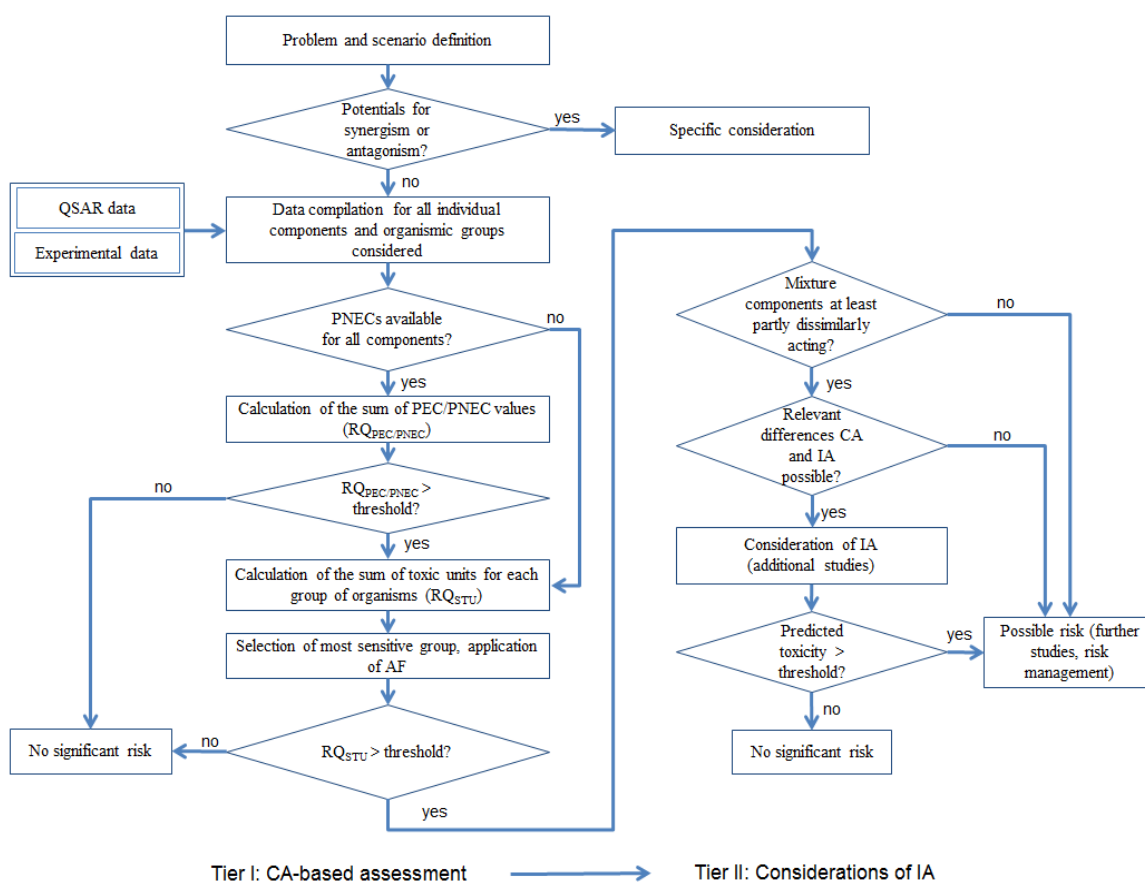Tier I: CA-based assessment → Tier II: Considerations of IA

Figure 4: Suggested two-tiered outline for predictive ecotoxicological risk assessment of chemical mixtures, figure redrawn with minor amendments from Backhaus and Faust (2012).

9. Environmental regulation approach on mixture effects

The scientific aspects of combined effects of chemical mixtures need to be connected to chemical regulation frameworks. Syberg et al. (2009) aimed to demonstrate how mixture toxicity assessment can be more thoroughly integrated into existing European chemical regulations (REACH and Water Framework Directive, WFD). They concluded that it is feasible to integrate a mixture toxicity approach into both legislations. CA, they suggest, should be applied as a default model for assessment of combined toxicity, with use of a PEC/PNEC based cut-off value for individual contaminants of PEC/PNECs > 0.1, and that required toxicity information should be made available by the construction of a database that includes data on chemicals in the European environment. They also suggest that REACH and WFD only should include combined toxicity assessments in specific situations. In REACH, which is principally based on evaluations of single substances, manufacturers and importers of chemicals are required to gather and register toxicity

information and other properties of each substance, which will allow their safe handling. These assessments should include safety margins to take account of effect uncertainties, which can be seen as a passive way to accommodate the possibility of combined effects of pollutant mixtures. However, there have been expressed concerns in the EU system that this safety margin approach may not provide sufficient security and that the risk related to chemical mixture effects should be addressed in a more systematic way. The WFD and the Marine Strategy Framework Directive (MSFD), take a similar approach as REACH, by having their regulatory focus on the chemicals being present in the mixture, and depending on which toxic properties these chemicals might have individually (Borja et al., 2010; Fuerhacker, 2009). The contaminant part of the required assessments system to achieve the "good water status" or "good environmental status" of water masses has basically focused on agreements on toxicity classifications criteria and Maximum Permissible Concentrations (MPCs) for individual contaminants. According to the WDF, all inland and coastal waters within EU should achieve ''good status'' by 2015, and the article 16 of the WDF describes how and by when Environmental Quality Standards (EQS) for pollutants should be developed. In this connection, the term EQS is defined as the concentration of a particular pollutant or group of pollutants in water, sediment or biota which should not be exceeded in order to protect human health and the environment. Pollutants that represent a significant risk should be identified and classified as priority substances by the European Commission, and the most hazardous of these should be classified as priority hazardous substances. In 2008, a separate directive (directive 2008/105/EC) was approved to establish EQS limits for 33 priority substances and 8 priority hazardous substances in surface waters. The same directive also introduced generic EQS limits for a small number of these priority substances (hexachlorobenzene, hexachlorobutadiene and mercury) in sediment and biota. Although these guidelines represent established approaches for single chemicals, the key question remaining to be answered is whether they may also offer an efficient approach for the evaluation and regulation of combined toxicity phenomena. Leung et al. (2005) and Bjørgesæter (2009) used field-based species sensitivity distributions (f-SSD) for more than 600 sediment-living marine animal species in their natural environment to calculate EQS values for heavy metal and PAH contaminants being present in sediments around offshore petroleum fields. They found these EQS values to be 8-33 times lower than the current Norwegian EQS values which have been derived in the

standard way from toxicity test on individual chemicals. On the other hand the heavy metal EQS values corresponded well with those developed through equilibrium partitioning by Altin et al. (2008) for the same sediment ecosystems, and which also to some extent encompassed combined effects. An individual chemical approach may therefore result in EQS values that are strongly under-protective.

There are active processes within the EU system aimed to develop ERA approaches and tools capable of incorporating combined effects. One relevant EU process that in 2004 was started within the sixth framework programme was the NoMiracle (NOvel Methods for Integrated Risk Assessment of CumuLative stressors in Europe) project which was aiming to improve both human and environmental risk assessment procedures by addressing major shortcomings of current ERA approaches. The outcome of the study includes novel ERA tools and these have been made available at the internet (http://nomiracle.jrc.ec.europa.eu). Results from the project have also been reported in several articles, such as by Pistocchi et al. (2011) who presented novel cumulative risk mapping methods making use of a CA approach to pesticides, and Løkke (2010) who described novel tools to analyse, characterize and quantify the combined risks of multiple cumulative stressors addressing both mixtures of chemicals alone or in combination with biological or physical environmental factors such as pathogens and climate extremes.

10. Conclusions

To evaluate the potential hazards of chemical mixtures represents a most difficult challenge in connection with ecotoxicity research, environmental risk assessment and for regulatory toxicology. As discussed in this paper, a broad range of anthropogenic contaminants (and animal species) are thought to be involved in combined toxicity phenomena. However, it's highly likely that combinations of compounds and compound groups that have specific (and similar) MoA, high potency and wide-spread usage which contributes to locally high exposure concentrations, represent the greatest risks for aquatic organisms in connection with combined effects. Furthermore, compounds that affect especially sensitive life-stages or organisms, and compounds that interact with toxicity targets being conserved across multiple taxa, may also be of particular concern. Animal species which are in high ecological/trophic positions that make them biomagnify persistent organic pollutants (POPs) are generally at risk for mixture effects. In addition to

chemical contaminants, a broad range of non-chemical factors may potentially influence or interfere with combined stress situations in organisms at risk. Current research on combined toxicity and multiple stressors focuses on developing and defining detailed adverse outcome pathways (AOP) to provide insight into mechanisms and modes of action being relevant for combined toxicity. Contaminants and mixtures which have MoA and AOP that conceptually link them (directly or indirectly) to disruption of biological fitness (e.g. growth, development and reproduction) will likely be of highest priority in mixture effect research, environmental risk assessment and in chemical and environmental regulations. The ERA of chemical mixtures involving a tiered approach and CA based mixture toxicity assessments as the first tier appears currently as feasible based on the available chemical toxicity information and the existing regulatory frameworks for chemicals and effluent releases to aquatic environments in Europe (e.g. REACH and WFD). However, as better data and analyses on multiple stressor and combined toxicity situations will emerge, a better detailing of effect mechanisms and effect predictions can be expected, and the methods for evaluating environmental and health risks of combined effects can be improved.

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