The SOLUTIONS project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no. 603437

Solutions for present and future emerging pollutants in land and water resources management

THEME

ENV.2013.6.2-2

Toxicants, environmental pollutants and land and water resources management

Start date of project: 1st October 2013 Duration: 5 years

External Deliverable 9.1

Guidance document and decision tree for use of chemical, bioanalytical, and ecological tools in RBSP identification, impact assessment, and establishment of cause-effect relationships

---

Due date of deliverable: March 2018

Actual submission date: 31.05.2018

Author(s)

Rolf Altenburger (UFZ), Wibke Busch (UFZ), Miren López de Alda (CSIC), Martin Kraus (UFZ), Beate Escher (UFZ), Andreas Focks (Alterra), Werner Brack (UFZ)

Revision [1]

Dissemination Level		
PU	Public	x
PP	Restricted to other programme participants (including EC)	
RE	Restricted to a group specified by the consortium (including EC)	
CO	Confidential, only for members of the consortium (including EC)	

1.1 Summary

Environmental monitoring of chemical contamination of water resources provides a knowledge basis for safeguarding the environment against adverse biological effects of anthropogenic chemical contamination. While current regulatory efforts focus on monitoring and assessing a few legacy chemicals, chemicals occur in larger variety and in mixtures in the aquatic environment. The international, EU-funded project SOLUTIONS, explored several routes to more adequately address the challenges of mixture contamination by inventing, advancing, and improving chemical compound analysis and complementary bioanalytical effect detection methods.

First of all, several water sampling methodologies were developed and advanced to improve analyte enrichment prior to analysis. This provides improved analytical sensitivity, access to time integrated exposure assessment and allows higher sample volume enrichment for subsequent bioassay studies.

Secondly, multi-residue target analysis methods were advanced to cover an extended range of anticipated chemicals co-occurring in the environment. Moreover, by establishing automated sample pre-processing and improving sensitivity and detection limits for known bioactive compounds of concern and through novel analytical chemistry methods, multiple components of potential emerging concern could be monitored and used to characterize source related contamination patterns.

Thirdly, the consortium advanced, adapted and quality controlled an array of bioanalytical methods for complementary use in an effect-based water monitoring. Provision of standard operating procedures for various assays, performance characteristics for compound and environmental samples, and dedicated mixture studies for combined effect detection provide substantial progress towards routine application.

The established sampling and detection methods were used for the identification of river basin specific pollutants, assessing the impact of mixture exposure, and improving causality between exposure and adverse effects.

Strategies for identification of river basin specific pollutants of potential concern were furthered by complementing chemical analytics with non-target screening techniques and approaches for automated structure elucidation of suspect signals. Furthermore, a virtual effect-directed analysis approach was elaborated which builds on correlating and stepwise reducing multivariate chemical compound and biological effect information.

For assessing the impact of mixture exposure in freshwater, bioassay panels were compiled and tested with single chemicals, designed mixtures and environmental samples. The effects in the designed mixtures were compared to component-based mixture effect prediction indicating that the mixture concept of concentration addition is a useful default assumption and that effects can be observed against a background of non-specifically acting chemicals. This mixture effect concept forms the basis for modelling of complex mixtures of known and ill-defined composition. Furthermore, the derivation of effect-based trigger values, would allow stand-alone effect-based surface water contamination

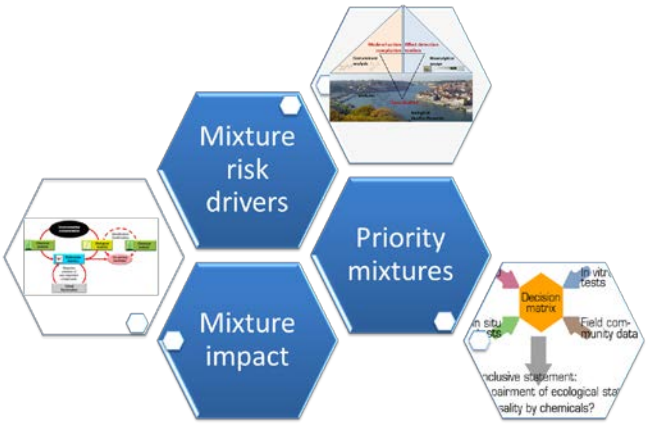
assessment. A proposal for this was tested using different case studies. Moreover, chemical and ecological observations in river monitoring were studied by correlation analysis to explore the potential of linking chemical status with ecological effects.

In order to improve the causality between chemical exposure description and biologically adverse effects, iceberg modelling, which balance chemical and biological equivalent concentrations, and higher tier iterative effect-directed analysis were studied in case studies demonstrating the potential of these approaches. To assess the plausibility of chemical-induced ecological effects an approach using multiple lines of evidence was developed. It is suggested as a strategy to harvest all available information in a structured and transparent approach for linking chemical contamination and ecosystem level effects.

The different methodologies and approaches were explored using dedicated case studies at the Danube, Rhine river basins and for rivers of the Iberian Peninsula and the results were used in various contexts, e.g. the identification of RBSPs. This deliverable provides a synthesis of all findings to demonstrate guidance for future solution-oriented environmental monitoring. It explores the scope for more systematic ways to assess mixture exposures and combination effects in future water quality monitoring.

1.2 [publishable] Graph

Please prepare a graph as simple as possible depicting the deliverable.



2. List of Contents

3. List of Abbreviations

APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
BEQ	Bioanalytical equivalent concentration
EI	Electron ionization
EDA	Effect directed analysis
EBM	Effect based method, bioassay
EBT	Effect based trigger value
ESI	Electrospray ionization
EQS	Environmental quality standards
FTICR	Fourier transform ion cyclotron resonance
GC	Gas chromatography
HPCCC	Hydrodynamic counter current chromatography
HPLC	High pressure or high performance chromatography
HRMS	High resolution mass spectrometry
LC	Liquid chromatography
LOD	limit of detection
LOE	Line of evidence
LOQ	Limit of quantification
LVSPE	Large volume solid phase extraction
MOA	Mode of action
MS	Mass spectrometry
QuEChERs	Quick, easy, cheap, effective, rugged, and safe
RBSP	River basin specific pollutants
REF	Relative enrichment factor
RiBaTox	SOLUTIONS guidance tool for River Basin Toxicants
SOP	Standard operating protocol
SPE	Solid phase extraction
STU	Sum of toxic units
LC	Liquid chromatography
UHPLC	Ultra high pressure or high performance chromatography
WOE	Weight of evidence
WFD	Water framework directive

SOLUTIONS project partner abbreviation list

UFZ Helmholtz Centre for Environmental Research GmbH; **Deltares** Foundation Deltares; **EI** Environmental Institute; **IVL** Swedish Environmental Research Institute; **JRC** Institute for Environment and Sustainability -IES- of the European Commission's Joint Research Centre; **KWR** KWR Water BV; **Eawag** Swiss Federal Institute of Aquatic Science and Technology; **NIVA** Norwegian Institute for Water Research; **LMC** Laboratory of Mathematical Chemistry - Asen Zlatarov University Bourgas; **OEKO** Oeko-Institut e.V. - Institute for Applied Ecology; **SU** Stockholm University; **RIVM** National Institute for Public Health and the Environment **ALTERRA**; **CSIC** Agencia Estatal Consejo Superior de Investigaciones Científicas; **F+B** Faust & Backhaus Environmental Consulting; **Brunel** Brunel University; **VITO** Flemish Institute for Technological Research; **UNOL** Carl von Ossietzky University Oldenburg; **WatchFrog**; **UNS** University of Novi Sad; **MAXX** MAXX Mess- und Probenahmetechnik GmbH; **INERIS** Institut National de l'Environnement Industriel et des Risques; **UU** Utrecht University; **Synchem** Synchem UG & Co. KG; **UOB** University of Birmingham; **ULIV** University of Liverpool; **ICPDR** International Commission for the Protection of the Danube River; **DynEx** Dynamic Extractions; **NJU** Nanjing University; **RWTH** RWTH Aachen University; **MU-RECETOX** Masaryk University - Research Centre for Toxic Compounds in the Environment; **Mermayde**; **IPB** Leibniz Institute of Plant Biochemistry; **HAM** Hammerbacher GmbH Consulting & Facilitation; **UGOT** University of Gothenburg; **KOCMOC** KOCMOC.NET Design Agency; **UB** University of Bern; **UQ** University of Queensland; **UNICAMP** University of Campinas.

1. Scope for the guidance document on methods for contamination assessment

The ambition of this ‘guidance document and decision tree for use of chemical, bioanalytical, and ecological tools in RBSP identification, impact assessment, and establishment of cause-effect relationships’ (SOLUTIONS deliverable 9.1) is to synthesize and reflect the various efforts undertaken in the SOLUTIONS subproject Tools which focussed on experimental and observational methods useful for water quality determination. The context is given by the goal of the EU water framework directive (2000/60/EC) to achieve a good chemical status for European water bodies. From a scientific perspective the quest to monitor a chemical status comprises of various challenges if a comprehensive account of today’s complex chemical contamination of water bodies and their potential adverse outcomes is to be acknowledged (Brack et al. 2015). To develop strategies and advancing means for developing future water monitoring the SOLUTIONS project covered critical aspects to deal with the various challenges.

On the side of detection of contaminant occurrences in water we advanced, explored, adapted and quality controlled a range of chemical and bioanalytical techniques. The major technical problems in doing so comprised of compound detection below concentrations defined by environmental quality standards (EQS), estimation of time-averaged concentrations, more adequate coverage of environmental mixtures, detection and unraveling of internal contamination of biota, availability of comparable standard operating protocols, robustness checks, quality controls and standards for novel techniques or applications. More details on the initial planning and derivation of strategies can be found in Altenburger et al. (2015).

On the side of providing guidance on the use of state of the art methods for contamination assessment in water bodies we studied various tools and explored combinations of them in specifically dedicated case studies in the river basins of the Danube, Rhine, Iberian rivers and the river Elbe. Here, we reflect the newly generated knowledge on experimental tools and their potential future role as problem-solving strategies with respect to the identification of RBSPs, the assessment of biological impacts and the establishment of cause-effect relationships.

The following chapters will illustrate these arguments. First, we illustrate major achievements and promises of methods tested for their potential to contribute to an advanced diagnosis of the status of chemical contamination and subsequently, we discuss problem-solving strategies for various questions. We also reflect on the case studies outcome and provide systematic access to the decision tree that is to be used in the SOLUTIONS expert system RiBaTox.

2. Methods for an advanced diagnosis of the status of chemical contamination of freshwaters

In the following chapter we summarize the findings of the SOLUTIONS project regarding chemical sampling methods, chemical analytical techniques, and bioanalytical methods.

2.1 Sampling methods

Sampling and sample preparation is one of the most important steps in any (bio)analytical method. The objective of sampling is to collect a portion of material from an environmental compartment small enough in volume to be transported conveniently and handled in the laboratory, while still adequately representing the part of the environment sampled. “Representativity” is a key word in this understanding, not only in terms of whether the portion of the sample truly represents the natural environment sampled, but also whether the sampling and following sample handling is under sufficient control that no changes (contamination, loss) occur.

Among the different types of sampling, grab (or spot) sampling is likely the most often used under the Water Framework Directive for ecological and chemical status monitoring (Quevauviller et al. 2008). Grab sampling is the most basic form of sampling. It may be carried out in all compartments, including water and biota, and it involves a sampling device (sampler, pumping system, nets …) that collects a sample at a given location and time. Hence the samples, in principle, are representative only for these conditions. If a water body is anticipated to be more or less homogeneous (in space and time), these samples may provide this expected information.

In fluctuating systems, however, like rivers or water treatment plants, where the quality of the water can change rapidly, integrated sampling is an option to avoid arbitrary findings and missing of contamination peaks. Integrated sampling, covering a given transect or area and/or period of time, may consist of a series of grab samples that are collected and pooled either manually or with the aid of specific equipment, or of continuous constant or flow proportional collection by pumping systems. In the first case, collection can be time-, flow-, or volume-proportional, and the higher the sampling frequency the more representative the information provided will be (Ort et al. 2010).

Apart from grab and integrated sampling, other sampling strategies, developed and explored in depth in the SOLUTIONS project, are passive sampling and large-volume solid phase extraction (SPE). These strategies, together with those followed for advanced sample preparation based on the use of high performance counter current chromatography (HPCCC) and on-line pre-concentration techniques, are discussed in the following sections, since these strategies have been in the focus of the SOLUTIONS project as potential analytical technical solutions for improvement of limits of detection (LOD) and quantification (LOQ) of the methods. This would hence foster high-sensitivity analysis of toxic contaminants.

Passive samplers

Passive sampling is a powerful tool that can conveniently be used for monitoring of organic compounds in water and other environmental compartments. It has been designed to provide estimates of freely dissolved concentrations of chemicals which have been shown to be in many cases appropriate to explain exposure and adverse effects in biota. Partition-based passive sampling also allows direct comparison of measured water concentrations with concentrations in diverse compartments (e.g. water, sediment, biota), based on the assessment of chemical activity in those matrices. The time-integrating character of sampling in combination with the application of a sampling matrix (e.g. polymers) with well-defined and constant properties makes it possible to achieve a lower inherent variability of exposure information compared to traditional grab sampling of water, and is thus suitable for assessment of pollutant average concentration trends in water bodies. A specifically dedicated deliverable of the SOLUTIONS project (see

Deliverable 10.1., abstract in Annex A) provides practical guidance on the use of passive samplers for monitoring organic pollutants in water. Two different categories of passive samplers are addressed. The first category comprises partition-based samplers for the measurement of non-polar contaminants, and the guidance sets focus on silicone rubber based samplers, which have been widely applied in the SOLUTIONS project. The second category encompasses adsorption passive samplers for monitoring of more polar aquatic contaminants. Herein typically applied samplers are based on the use of sorbents designed for solid-phase extraction. The guidance provided may assist users of passive samplers, who wish to implement passive sampling methods in their research or monitoring work, as well as more experienced users in the use of the available methods according to the state-of-the art. The guideline addresses principles of passive sampling, sampler preparation, field deployment, laboratory processing, chemical analysis, calculation of aqueous concentrations and associated uncertainty considerations. Aspects of quality assurance are also addressed. Finally, practical examples of sampler operation and sample processing procedures are provided, which have been developed and applied within the SOLUTIONS project.

Hydrodynamic counter current chromatography (HPCCC)

HPCCC is a chromatographic separation technique based on the partition of compounds between two immiscible liquid phases where the liquid stationary and mobile immiscible phases interact in a hydrodynamic force field within a helically wound coiled column to enable the separation. The stationary liquid phase is held within the column by centripetal acceleration forces enabling the mobile phase to be pumped through the column: A significant benefit of this technology (Dynex) is that through designing it to operate at the relatively high centripetal acceleration forces (or g field) it can be used in many different operational modes with compounds having a wide range of polarity, without disrupting the stationary phase. This versatility enabled the Brunel University team to explore a wide range of options in column specification and operational processing to develop an extraction method for pollutants at very low concentrations contained in large volumes of water across a range of polarities of the analytes of interest. In the method(s) developed, the mobile phase is the water sample e.g. river water, potentially containing the pollutants; the stationary phase is an appropriate organic solvent system. A further advantage of the Dynex hydrodynamic HPCCC technology is that the column works at low pressure using relatively wide bore tube which results in important benefits: The column length (within reason) is not limited; increasing column length improves resolution which in turn enables the separation of a complex mixture of compounds. A second important aspect of this wide bore hydrodynamic column is that it can deal with particulate contamination (providing the particulates are not physically greater than the diameter of the tube) and so it is therefore possible to take raw river water without the need for any pre-treatment (other than physical sieving to remove large particles) and perform a separation. Further, since both phases of this separation technique are a liquid, it enables the total recovery of every compound separated into either phase to be recovered without loss, analysed, recycled or collected for further processing and so when the stationary phase becomes saturated and needs replacing it is simply a case of emptying the column and refilling with fresh solvents.

In subsequent work, the application and use of HPCCC as part of a pollutant monitoring toolkit for SOLUTIONS will be determined. It should be stressed, however, that this is a concentration and sampling tool and although it is possible to monitor all outputs from the processing column using the detector systems and collect these as discrete fractions, separate downstream analytical systems are required to monitor specific analytes.

We see two future applications for the Dynamic Extractions HPCCC system which take advantage of the unique features of the technology and which will be verified once the work is completed: One is a continuous sampling, extraction and concentration tool which can be located at any strategic location in the field to monitor pollution. The second is a laboratory-based tool operated as a “batch process” in a similar way but using large grab samples from the water course. Detailed information on the HPCCC

variables tested and methods developed within SOLUTIONS can be found in Deliverable 10.1.

Time-integrated sampling by *in situ* large volume solid phase extraction

Establishing targeted and non-targeted chemical screening analysis in combination with *in vitro* and i*n vivo* assays is an essential step for a more holistic monitoring of emerging contaminants in water. However, such an approach requires on site collection of very large sample volumes. Large volume solid phase extraction (LVSPE) is a recently developed instrument that can support this approach since it allows automated and composite sampling of water resources for all purposes of effect based monitoring and toxicant identification. It is also usable in combination with chemical analysis. The device and method can be tailored to the specific needs and goals of the sampling campaign and monitoring program. It is possible to run LVSPE to collect a large volume sample in short-term over some hours on site, but also to gain a time-integrated large-volume sample over longer durations (e.g. 7 days) including the frequent collection of sub-samples. In comparison with grab sampling, classic automated and passive sampling, LVSPE has the following advantages:

1. Time-integrative sampling ensures improved representativeness of the sample in terms of baseline and peak loads of chemicals;
2. Within the principal limitations of solid-phase extraction, LVSPE ensures a comprehensive sampling of the complex contamination of water bodies including known and unknown organic chemicals with minimal bias and discrimination;
3. The binding of the compounds to the solid phase prevents degradation;
4. The exact volume of water extracted can be calculated;
5. On-site extraction reduces logistic, technical, economic and scientific issues related to the storage and transport of large water volumes to the laboratory and subsequent processing.

LVSPE systems have been designed for collection of sample volumes from 50 L of up to 1000 L of water. Details on their main characteristics and field applications within SOLUTIONS can be found in Schulze et al., 2017 and in Deliverable 10.1. LVSPE was successfully applied in the SOLUTIONS project within the Danube and Rhine River Basin case studies for the purpose of effect-based and chemical analyses (Muz et al. 2017, Tousova et al. 2017, Neale et al. 2015). The work performed demonstrated that LVSPE is a useful tool for the high quality sampling and extraction of pollutants for chemical and effect-based screening of water resources in field applications. LVSPE allows for on-site extraction of large volumes of water from natural or artificial water sources and thus provides sufficient sample volumes at higher enrichment factors required for chemical and biological screening especially if a set of different bioassays is deemed necessary. Furthermore, LVSPE appears to be suitable for the enrichment of complex mixtures of known water contaminants with only little systematic dependence from physicochemical properties and with reasonable recoveries (Schulze et al. 2017). The effect recovery in bioassays was also satisfactory and process blanks were shown to be of sufficient quality (Neale et al. 2018). The flexible concept of the device allows tailoring the configuration according to the user's specific needs. The device will facilitate the development of effect-based and chemical assessment strategies to supplement the existing concepts of water quality assessment. For example, the samples can be subjected to a screening in a broad set of bioassays and subsequent use for effect-directed analysis in specific assays to unravel cause-effect relationships for the prioritization of effects and pollutants.

On-line extraction and clean-up methodology for LC

As a recent trend, analysts and analytical technology developing enterprises are investing considerable resources in the development of green, fast and high throughput analytical approaches to determine the occurrence of emerging organic contaminants in the environment, without compromising the sensitivity, accuracy, precision, and selectivity of the methodology used. On-line solid phase extraction coupled to liquid chromatography mass spectrometry (SPE-LC-MS) has been proved to be a very versatile and

reliable technique for on-line sample pre-concentration and extract clean-up. The column switching configuration is the most extended one for on-line coupling of the sample preparation process to the LC-MS system. It is basically a bi-dimensional system in which a switching six- (or more) port valve interfaces two dimensions or columns allowing the transfer of the analytes from the first column where the preconcentration or the clean-up process takes place to the second column where they are LC separated before entering the MS detector (Fumes et al. 2017). The sample treatment process occurring on the first dimension is directly related to the sorbent or column operated in there, with solid phase extraction (SPE) and turbulent flow chromatography being the main sample preparation techniques used in the column switching approach. On the other hand, on-line methodologies where preconcentration of the samples is carried out with automatically replaceable SPE cartridges with systems like SymbiosisTM or Prospekt-2TM can only use high performance liquid chromatography (HPLC) for separation of the analytes due to the high back pressure that the use of ultra-high performance liquid chromatography (UHPLC) columns would generate in the system. Thus, combination of UHPLC with on-line preconcentration of environmental samples is subject to the use of not automatically replaceable preconcentration columns, coupled on-line with the chromatographic column via techniques like EquanTM and Agilent dual column systems.

On-line SPE in the environmental field has been successfully applied for the extraction and pre-concentration of emerging contaminants dissolved in aqueous matrices, and also for the clean-up of sample extracts of high complexity. In any case, as the liquid sample passes through the SPE sorbent, analytes of interest are retained whereas undesired matrix components elute, according to the physical-chemical interactions among the sorbent and the sample components.

One of the objectives within SOLUTIONS was to develop new on line SPE-LC-MS/MS methods for analysis of relevant classes of emerging pollutants in environmental samples and to demonstrate their performance in comparison to off-line methods, particularly in terms of sensitivity. Also, their robustness and value of applicability was investigated in different case studies.

On-line methodologies procure in general better sensitivity, recovery and precision than off-line methodologies and can be used in principle for all LC-MS amenable emerging contaminants, including polar and ionic ones. Their usefulness in numerous environmental applications including the analysis of pesticides, perfluoroalkyl substances, illicit drugs, chemotherapy agents, and various other classes of pharmaceuticals in water samples, has been broadly demonstrated by the CSIC partner in the frame of SOLUTIONS. The methods developed as well as applications of them in different rivers and other aquatic compartments have been described in detail in several scientific publications (Mendoza et al. 2014, Palma et al. 2014), (Isidori et al. 2016), (Schwanz et al. 2016) (González-Alonso et al. 2017)), and some of these methodologies can be found as SOPs in Deliverable 10.1.

2.2 Chemical analytical methods

Combinations of gas chromatography (GC) or liquid chromatography (LC) with mass spectrometry (MS) are typically the methods of choice for a wider target analysis of emerging contaminants in water and biota samples due to the provided sensitivity and selectivity. LC-MS and GC-MS cover a large and overlapping fraction of the chemical domain of known emerging and legacy contaminants, but there is no single technique able to address all targeted compounds. Furthermore, different ionization techniques in LC-MS have widely varying ionization efficiencies for different compound classes. The choice between GC-MS and LC-MS is determined mainly by the volatility and hydrophobicity of the compounds. Large shares of the more hydrophilic compounds which typically are relevant for water are LC-MS amenable (e.g., current-use pesticides, pharmaceuticals, industrial chemicals and their transformation products). On the other hand, many hydrophobic and bioaccumulative compounds are typically GC-MS-amenable, yielding thus a more prominent role for this technique in the analysis of aquatic biota.

In the past, mainly GC-EI-MS on quadrupole instruments and LC-ESI-MS/MS on triple quadrupole

instruments were most commonly applied for the analysis of emerging contaminants, while the analysis of polychlorinated dioxins and furans was a niche application of GC coupled to high resolution magnetic sector instruments. However, in the last years, an increasing number of methods were established based on high resolution mass spectrometry (HRMS) using time-of-flight (ToF) or Orbitrap MS, or in rare cases Fourier transform ion cyclotron resonance (FTICR)-MS (e.g. (Myers et al. 2014)).

A range of conceptually different analytical strategies have been developed for the monitoring of emerging contaminants. In principle, we differentiate between target, suspect, and non-target analysis or screening. A clear distinction between target analysis and target screening methods is not at hand. Typically, target analytical methods focus on a limited number of compounds, and the sample preparation and analysis has been particularly optimized for these compounds, and the removal of matrix interferences. In contrast, target screening methods strife to include a larger number of compounds in one analytical run, typically more than one or two hundred. Thus, the sample preparation is often limited in order to allow covering a wide range of physicochemical properties by avoiding steps resulting in a removal of compounds. Thus, it is also easier to include new compounds of interest into an existing target screening method.

Suspect screening strategies (Krauss et al. 2010, Moschet et al. 2013) aim at detecting known compounds in the absence of reference standards, thus retention times and mass spectra are unknown. This approach starts from the known structure and molecular formula, from which the masses of the ion to search can be calculated considering the respective type and polarity of ionization (e.g., electrospray ionization typically forms protonated or deprotonated molecules). These ions are than searched for in the MS data in a first step, either based on extracted ion chromatograms of these ions or in peak lists from automated peak detection algorithms as used for non-target screening (see below). Positive findings require subsequent confirmation steps which are based on a comparison of observed and theoretical isotope patterns as well as of measured and in silico predicted MS/MS spectra or retention times.

In contrast to suspect and target screening, non-target screening in a strict sense starts solely from the analytical data without any knowledge of the compounds present. It typically begins with a peak detection (also called peak picking) step, which finally results in a list of all detected peaks and usually includes a removal of peaks that are assigned to a laboratory background and to blank samples. For this procedure, several commercial and vendor-specific software packages are available. It is obvious that the selectivity of high resolution MS (HRMS) offers a clear benefit over low resolution MS, making the former the instruments of choice for non-target screening. Typically, peak lists from HRMS data of environmental samples contain several thousand peaks. Thus, a prioritisation for the peaks of interest is the next step, which can be based on peak intensity, frequency across a set of samples or other criteria depending on the purpose of the study. For prioritized peaks, two different pathways may follow; either spectral library search or molecular formula determination, the choice of which depends to a large extent on the ionisation technique chosen.

Electron ionization can be easily standardized and results in a strong fragmentation of the molecular ion, which may even become be absent due to complete fragmentation. The obtained mass spectra are highly reproducible and rich in structural information, which resulted in the development of large mass spectral libraries (e.g., NIST Library for Mass Spectrometry and Wiley Registry of Mass Spectral Data with hundreds of thousands of spectra). Based on this fact, GC-EI-low resolution MS on quadrupole instruments has been the main method for successful non-target screening for a long time. In the case of compounds not being present in a library, non-target screening based on EI-MS data is often difficult and involves manual interpretation of the spectra, supported by computer-assisted structure elucidation methods. The uncertainty about the presence of a molecular ion, however, remains a major obstacle also for application of EI-HRMS.

Soft ionisation techniques like ESI, APCI and APPI, by contrast, often leave the parent ion intact and the fragmentation may be limited. Thus, MS/MS techniques have to be used to obtain fragment ions diagnostic for a structure, but fragmentation patterns differ among different instruments and

fragmentation energies used. While MS/MS libraries are rapidly growing, they are currently still of limited use. The main approach within non-target screening is therefore the determination of molecular formulas for the compounds of interest using accurate mass and isotope pattern information. Subsequently, candidate structures for these molecular formulas are searched in large chemical compound libraries (such as ChemSpider or PubChem), and the retrieved candidate list have to be ranked and filtered for the most probable structure. This ranking is based on a comparison between predicted properties of each structure with the observed experimental information. To this end, candidate selection workflows have been developed including MS/MS fragmentation prediction, retention time prediction, and commercial relevance of a compound (Gago-Ferrero et al. 2015, Ruttkies et al. 2016). Ideally, these workflows result in a low number of likely candidates, for which reference standards have to be obtained for a final confirmation.

The NORMAN Network published the Suspect List Exchange in 2015 (http://www.norman-network.com/?q=node/236) as a collection point for various "suspect lists", together with details behind each list (e.g. publication or data source) and a list of the chemicals involved as InChIKeys, a chemical exchange format, for integration in the non-target screening workflow with MetFrag (Ruttkies et al. 2016) (http://c-ruttkies.github.io/MetFrag/).Workflows to perform this in the open programming language R were developed within SOLUTIONS (package "RChemMass", available on request) in collaboration with SOLUTIONS and NORMAN members, along with Antony Williams from the US EPA CompTox Chemistry Dashboard (https://comptox.epa.gov/dashboard/). The lists from the NORMAN Suspect List Exchange are being curated and integrated within the Dashboard, to further improve the exchange of information. Furthermore, the Dashboard is already connected to MetFrag to be used within a non-target workflow. The resources mentioned above have all been integrated within the SOLUTIONS non-target screening workflow (deliverable 10.1). Suspect screening was applied in the Rhine case study in collaboration with Stellan Fischer (KEMI), who has provided suspect lists for the NORMAN Suspect List Exchange, the CompTox Chemistry Dashboard and for fellow SOLUTIONS partners LMC and UFZ. The list provided to LMC and UFZ was curated with the MS-ready workflows in R and the Dashboard.

2.3 Bioanalytical methods

Biological effects of contaminants can be observed and quantified using various methods (Escher and Leusch, 2012). Typically, organism or cell responses are recorded following an exposure event. Exposure can be controlled and varyied in a lab exposure setting against defined compounds, mixtures, or environmental samples or less defined by employing biosystems in a given field situation. Biological effects can comprise various reactions, ranging from a molecular-molecular interaction, to the observation of an apical effect such as the growth of a population over time. It is usually compared to a control or reference system. In principle, effect detection may provide information on the type of exposure and or the potential for an adverse biological outcome. While effect detection does typically not require much sample pre-treatment, ultimate compound identification is not provided. Specific effect-based methods are already used for applications e.g. in food contaminant detection and in whole effluent assessment.

The SOLUTIONS project hypothesised that various effect-based methods, that can run short-term exposure of a few hours to days, could productively complement chemical analytical monitoring efforts to assess the quality of freshwater systems with regard to adverse impacts of contamination. Advancing effect-based methods for water monitoring purposes was pursued on the one hand by advancing and exploring a list of promising bioassays by studying their suitability for detecting either specific types of chemical action or observing integral apical effects for fresh water samples. From these efforts, we also compiled a modular panel of bioassays to cover different effect qualities/contaminants and suggested ways to use them as stand-alone methods. On the other hand toxicogenomic methods of effect-detection were developed and investigated to study whether the complexity of contamination patterns in environmental samples may be more comprehensively reflected by advancing off- and non-targeted effect-detection methods similar to non-target chemical detection. This line of thought was supported by

the evaluation of the modes-of-action of currently known water contaminants found in monitoring studies, which shows that many different molecular biological targets may be affected through the diverse chemical exposure (Busch et al 2016) while the proposed effect-based methods cover only a subset of their known modes of action (MOA) (Altenburger et al. 2015).

From the project efforts we learned that the several hundreds of distinct organic water contaminants that are currently detected in European freshwater systems may be aggregated into less than 30 MoA groups from a bioanalytical effect perspective (Busch et al. 2016). This offers scope for capturing mixtures of diverse contaminants via effect detection using effect-based methods (EBM). There is a substantial number of bioassays available for potential use in water monitoring both in the literature. In the SOLUTIONS consortium standard operating procedures for 36 bioassays were documented (deliverable 12.1, abstract in Annex C). The comparison between the knowledge on the modes of action of water contaminants and the effects detectable with the available bioassays shows that some prominent modes of action are covered by several assays e.g., endocrine or mutagenic effects. However, several known MoA, e.g. neuroactivity, are not captured by any of the currently available mechanism specific cell-based assays. Organism-based assays that detect apical effects, such as short-term studies on vitality and growth using fish eggs, daphnids and algae, may therefore help out if we are not dealing with delayed effects or system-specific mechanisms such as carcinogenicity or viral enzyme inhibition.

A non-target biological analysis that allows effect detection and diagnosis of the causes at the same time is still in a research stage and was explored focussing on transcriptomic signals following short-term exposures. We investigated bacterial, daphnid, and fish biosystems to reflect the different biological quality elements of pelagic systems and to capture the relation between biosystem-specific and unspecific chemical activity. Thereby, we applied toxicogenomic approaches with two major goals: a) to gain a deeper understanding of molecular processes in response to chemical stress and mixture exposures and b) to investigate the sensitivity and specificity of toxicogenomic methods for diagnostic purposes. A summary of the findings is documented in Annex F. The applications went as far as studying single chemicals, defined mixtures, and concentrated water samples, yet their use in routine monitoring is not yet in sight.

The SOLUTIONS case studies applications of cell- and organism-based bioassays for water monitoring showed that if 10-1000 fold pre-concentration of water samples was performed, bioassays were sensitive enough to detect effects of contaminants and discriminate between samples from different sites (König et al. 2017, Neale et al. 2015, 2016). The pre-concentration step requires additional quality control measures that needed to be further elaborated to render it an approach that could find consensus (Neale et al. 2018). In conjunction with advanced chemical analytical efforts it was furthermore demonstrated that bioassays captured exposure to mixtures rather than to individual component. From the differences between chemical analytical derived effect expectations and observed effects one might draw conclusions on compounds not captured in chemical analysis or on non-additive mixture effects. Differentiating between the two hypotheses is not straightforward as variance of the assay responses and precision of reported relative effect potencies is severely understudied.

The studies conducted by the SOLUTIONS' bioassay team and in collaboration with the NORMAN network provide a major leap forward in demonstrating the applicability of EBM for water quality monitoring. Most of the cell-based bioassays can now be run in a high-throughput mode with low volumes of extracts and larger numbers of samples, which renders them also more amenable for cost-efficient monitoring and applications such as EDA (Brack et al., 2016), where for sites that show unexpected effects the identity of the culprit can be identified by a stepwise fractionation and biotesting regime. An advantage is that the very same bioassays that can be used for water quality assessment can also be used for EDA (Hashmi et al., 2018). Various suggestions for the review of the water framework directive were derived with respect to accommodating for the use effect-based methods (Brack et al. 2017).

The efforts of the SOLUTIONS bioassay team also derived and rationalized a modular test battery

(Figure 1) and scrutinized it by single chemical testing (Neale et al. 2017), mixture evaluation (Altenburger et al., 2018), and by water quality monitoring applications (Neale et al., 2015; König et al., 2017; Neale et al., 2017) and EDA (Hashmi et al., 2018). The test battery designed in SOLUTIONS was designed and thoroughly validated by single chemical (Neale et al 2017) and designed mixture experiments (Altenburger et al. 2018) using chemicals that were shown to be relevant water pollutants covering diverse modes of action (Busch et al. 2015). All standard operating procedures were published (Neale et al. 2017) and quality assurance /quality control measures were thoroughly discussed in deliverable D12.2.

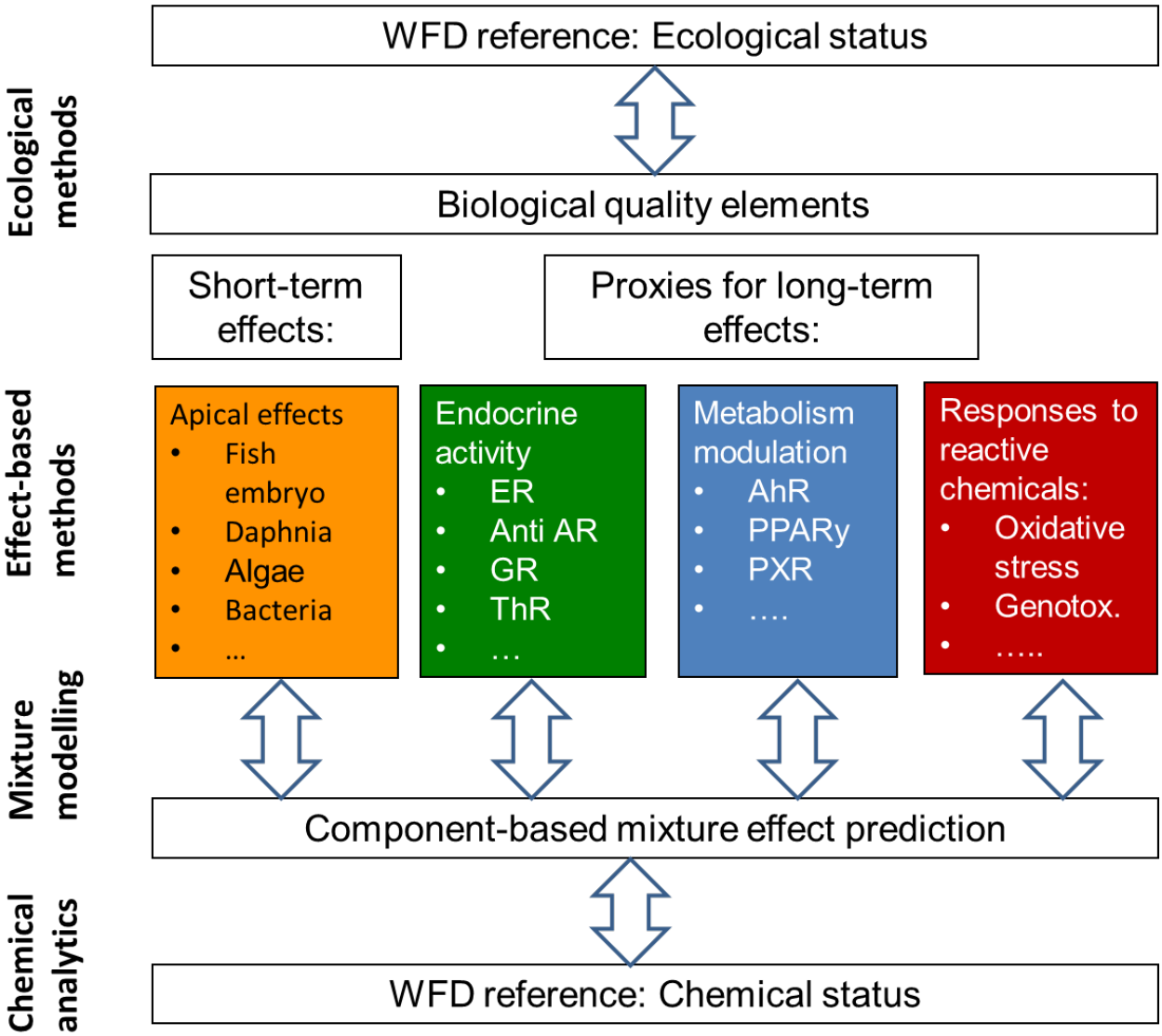


Figure 1. ***Panel of bioassay methods*** *for complementary effect monitoring covering several short-term and long-term biological effects (adapted from Deliverable D 12.2) in the WFD context*

There is even potential to use effect-based methods as stand-alone approaches in water monitoring provided one has assessment criteria for the observations detected. Whole effluent testing using bioanalytical methods as is done in the Germany under the national adoption of the WFD is an example of such a practise. The single chemical fingerprinting and mixtures studies also provided information for the derivation of effect-based trigger values (EBT) which would allow such a stand-alone use and

assessment of effect-based monitoring findings. The derivation of a generalised approach for EBT setting were based on the European environmental quality standards (EQS), on data generated in SOLUTIONS, and on literature data (Escher et al. 2018).

The test battery for surface water outlined in Figure 1 is also applicable for surface water for drinking water abstraction and for drinking water quality surveillance by adding additional assays for mutagenicity (as detected e.g. by the Ames test) and proxies for developmental effects(e.g. an extended fish embryo assay). The extended test panel was not applied as thoroughly to drinking water as to surface water, hence these and other anticipated modules of additional bioassays and their applications will need to be validated in the future. The various case studies outlined in more detail in Deliverables D12.1 and D12.2 demonstrated the complementarity of an analytical/bioanalytical approach for water quality assessment.

3. Problem solving strategies

This chapter undertakes to illustrate the application of chemical and biological methods discussed in the previous chapter for various purposes in water quality monitoring as they have been explored within the SOLUTIONS project. The water quality problems tackled address the challenges of identifying river basin specific pollutants, impact assessment for occurring chemical contamination, and the establishment of causal relationships between multiple contamination and deleterious biological effects. The strategies suggested and tested within the SOLUTIONS case studies typically comprise of targeted combinations of the previously discussed experimental techniques.

3.1 Identification of river basin specific pollutants

Strategies for identification of river basin specific pollutants (RBSPs) of potential concern were advanced by complementing chemical analytics with non-target screening techniques and approaches for automated structure elucidation of suspect signals. Furthermore, a virtual effect-directed analysis approach was elaborated which builds on correlating and stepwise reducing multivariate chemical compound and biological effect information.

RBSPs are pollutants considered of concern in a specific river basin as a result of the compilation and evaluation of both chemical and biological data from different sources. Apart from the priority pollutants included in the water framework directive, which are to be analysed in inland surface waters periodically and for which the results are reported to the European Commission (Directive 2013/39/EC), there are the pollutants included in a Watch List, which according to Commission implementing Decision (EU) 2015/495 also need to be scrutinized in order to decide whether on the basis of the newly generated information they should be included in the list of priority substances. Moreover, the so-called river basin specific pollutants (RBSP) are to be identified and monitored for specific river basins.

Identification of RBSPs can be conducted in different ways. The first and most commonly applied approach is target analysis. Target analysis, as discussed above, implies the a priori selection of the compounds to be investigated and the availability of suitable methods for their determination. In principle, the selection of the target analytes responds to available knowledge on issues such as the extent of use in an area of interest (e.g., pesticide use, consumption data in the case of pharmaceuticals, etc.), toxicity data extracted from the literature, and availability of suitable methods for their quantification.

Extent of use in the area and potential toxicity, if unknown, can be derived from prediction models, which are also useful for depicting future environmental scenarios. A train of linked models, which include sources and emission models, fate and transport modelling, substance metabolism and properties modelling, and human and ecological risk modelling, have been the focus of the SOLUTIONS activities on modelling (cf. Lindim et al. 2016) in order to estimate chemical occurrence in a spatially and temporally resolved manner.

Meanwhile, options for use of analytical methods for target chemicals determination depend on their local availability, performance (especially regarding sensitivity), and cost efficiency, and this obviously may bias the RBSPs identified in each case. Main potential constraints in this respect are analytical expertise, infrastructure, cost, and available budget.

To contribute to solve these problems and make progress in this matter, SOLUTIONS has worked on the development and further application of novel tools for integrated/large volume sampling and/or sample pre-concentration, namely, passive sampling, LVSPE, HPCCC and on-line SPE, which in general contribute to improve access to lower detection limits, larger sample extracts for multiple chemical and biological analyses, and also improved cost efficiency. These tools have been encompassed with the use of traditional and/or advanced GC/LC-(HR)(tandem)MS techniques to fulfil the main pursued analytical

objectives: an improved sensitivity to detect pollutants at levels below their PNECs, and better cost efficiency through the use of time-integrated sampling, advantageous sample extraction techniques (e.g., QuEChERs), and the development of multi-residue methods. All these techniques and methods, which have been designed and are provided as basic and important steps forward in the target analysis of environmental pollutants, are discussed and presented in detail in Deliverable D10.1 (abstract in Annex A). Apart from information on the basics, state-of-the-art, and further development of the previously mentioned sampling and sample pre-concentration techniques and on the suitability of using different GC- and LC-(HR)(tandem)MS techniques depending on the objective of the study, deliverable D10.1 provides standard operational procedures (SOPs) for making use of these tools, for analysis of more than 250 relevant (priority, Watch List, and other) pollutants in aqueous matrices and/or biota. These SOPs, which are provided as multi-residue methods for analysis of specific groups of pollutants, and as individual fact sheets for single compounds with indication of the main performance characteristics of the method for the given chemical, are compiled in annexes I to IV of the extended version of the deliverable D10.1. The same deliverable includes documentation on application of these tools and SOPs in different research works conducted in the SOLUTIONS river basin case studies.

Different RBSPs in water, sediment and biota were identified , for instance,in Iberian rivers by combining target chemical data (generated in part with SOPs described in deliverable D10.1 for analysis of different priority and emerging contaminant classes) and different risk assessment schemes that considered detection frequency, concentration levels and toxicity values from the literature (for details cf. SOLUTIONS deliverable D21.1).

Complementary to target pollutant analysis is the screening for suspect (metabolites and transformation products) and unknown pollutants. This approach, which expands enormously our vision for and capacity to comprehensively discover and investigate RBSPs in different scenarios, is a task that has shown an impressive development within the last years, and to which SOLUTIONS, as indicated in the previous sections, has contributed substantially. The SOLUTIONS Deliverable D10.1 includes in its section 6.2, the work-flow proposed within the project for identification of unknown compounds, of application in the identification of RBSPs, and the novel computational approaches developed for it (MetFrag2.2).

RBSPs can also emerge from the joint study of chemical (target, suspect or unknown) pollutants identified making use of the previously discussed tools and (eco)toxicological data gathered either locally or from literature. This kind of approaches which try to identify potential chemical agents causative of the effects observed has been applied in different case studies within SOLUTIONS. As an example, in a study conducted in the Iberian Rivers chemical occurrence data (and other environmental stressors derived from hydrological scarcity as e.g. interruption of the flow water regime or higher water temperature) were evaluated in conjunction with various biological descriptors associated with different trophic levels (macrophytes, phytoplankton, biofilms, benthic invertebrates, fish community) characterizing both ecosystem function and structure. The analysis pointed to organic microcontaminants as being only one cause of impairment, among others. For biological communities the effects of the multiple stressors may thus act synergistically and higher than those corresponding to their simple addition.

A further step in the identification of RBSPs involves the performance of EDA studies at different levels (from virtual EDA (vEDA) to higher-tier EDA (HT-EDA)) to unravel the pollutants that may be behind a certain observed adverse effect, and that may be either structurally identified or identified in a simplified approach using features like retention time and molecular mass. Guidelines and examples of the successful application of such approaches within SOLUTIONS can be found in deliverable D11.1 (abstract in Annex B).

As a novel approach, virtual effect-directed analysis (vEDA) undertakes to reduce the complexity of monitoring information through ‘virtual fractionation’. For this multivariate statistical techniques are applied to a larger set of environmental samples trying to correlate the chemical signals with observed effects. In contrast, in higher-tier EDA (HT-EDA), one or more fractionation steps are used to experimentally reduce the complexity of environmental mixtures to an extent that a limited number of

chemical signals finally remain in an active fraction, from which an identification and confirmation of the causative compound(s) is than possible.

In any study involving suspect and non-target screening, ultimate identification confirmation of a non-commercial pollutant suspected to be responsible for a given effect is, if possible, typically performed through the synthesis of the proposed structure (as indicated in section 6.2. of deliverable D10.1). Such standards can ultimately be used for analytical confirmation of suspect chemicals.

Finally, future scenarios building can help to provide indications on problems with respect to the anticipated impact of emerging RBSPs for the coming years.

3.2 Impact assessment

The ultimate goal of water quality management under the WFD is the provision of a good ecological and chemical status of European water bodies. It has been elaborated before that analytically undetected but toxicologically relevant compounds, transformation products and mixture effects may be overlooked in an approach that is purely based on chemical analytical measurements of pre-selected compounds. To balance possible gaps in chemical quality assessment, bioanalytical tools are suggested as a complement to improve an environmental impact assessments (CMEP 2014, Malaj et al. 2014, Altenburger et al. 2015).

The simultaneous exposure of organisms to different compounds may not necessarily mean that combined effects are evoked at detectable levels (Altenburger et al. 2004). To accommodate for this, bioanalytical tools that are tailored for specific mixture assessment objectives were developed and tested in SOLUTIONS (section 3.2.1) and approaches to quantify corresponding effect-based trigger values were developed (section 3.2.2, and deliverable 12.2, abstract in Annex D).

Finally, any approach to identify ecological impacts caused by chemical contamination on community composition has to discriminate the impact of toxic chemicals from non-chemical stressors, which often have a strong impact on community composition. A correlation-based approach which can statistically separate chemical- from non-chemical impact on variability within taxonomy- or traits based community composition was developed and tested in SOLUTIONS (Section 3.2.3, and deliverable 13.1, abstract in Annex E).

In the following, we will briefly explain the major progress made with regard to bioassay use in impact assessment, elude to the formulation of trigger values that would allow for stand-alone application of effect-based methods, and finally correlation-based efforts will be summarised that explored the potentials to directly link chemical contamination measurements with ecological information.

Effect detection using bioassay panels

For assessing the impact of mixture exposure in freshwaters, bioassay panels were designed and tested against component-based mixture effect prediction (Altenburger et al. 2015, 2018). The mixture studies demonstrated that the chosen bioassays capture mixture effects and that the concept of concentration addition is adequate for component-based mixture predictions. These mixture experiments also substantiate the concept of bioanalytical equivalent concentrations BEQ, which was applied in all SOLUTONS case studies to compare a biologically observed effect concentration(BEQbio) from bioassay experiments with a component-based predicted combined effect (BEQchem) based on equivalent concentrations, which are calculated from chemical analytical concentrations and the relative effect potencies of single compounds. Comparison of BEQbio with BEQchem allows the estimation of effects caused by unknown chemicals (iceberg model). The various case studies clearly demonstrated how useful the proposed test battery was to assess the water quality of surface waters in a more holistic way. Further details are laid out and discussed in the deliverable 12.1 (abstract in Annex C).

Effect-based trigger value derivation

A unified approach for effect-based trigger value derivation for bioassay-based environmental sample assessment was developed (Escher et al. 2018). This method is based on reading across from existing environmental quality standards AA-EQS of the European Union and some proposed national values. The proposed EBT are preliminary because only insufficient bioanalytical information is available for chemicals with existing EQS. More single chemical data must be obtained before a specific EBT can be considered final.

The derived EBT values were tested using the SOLUTIONS case studies and other literature studies. To this end, the literature data were grouped into untreated wastewater (WW) and stormwater (surface runoff) as one category of water that is likely not to meet the EBT and a second category of treated WW and surface water for which a different outcome was expected . While these categories cannot be assigned with a high precision and also we cannot be sure that untreated water is above the EBT and surface water below the EBT, the comparison shown in Figure 3 provides an idea about the range of effects observed in relation to the EBT.

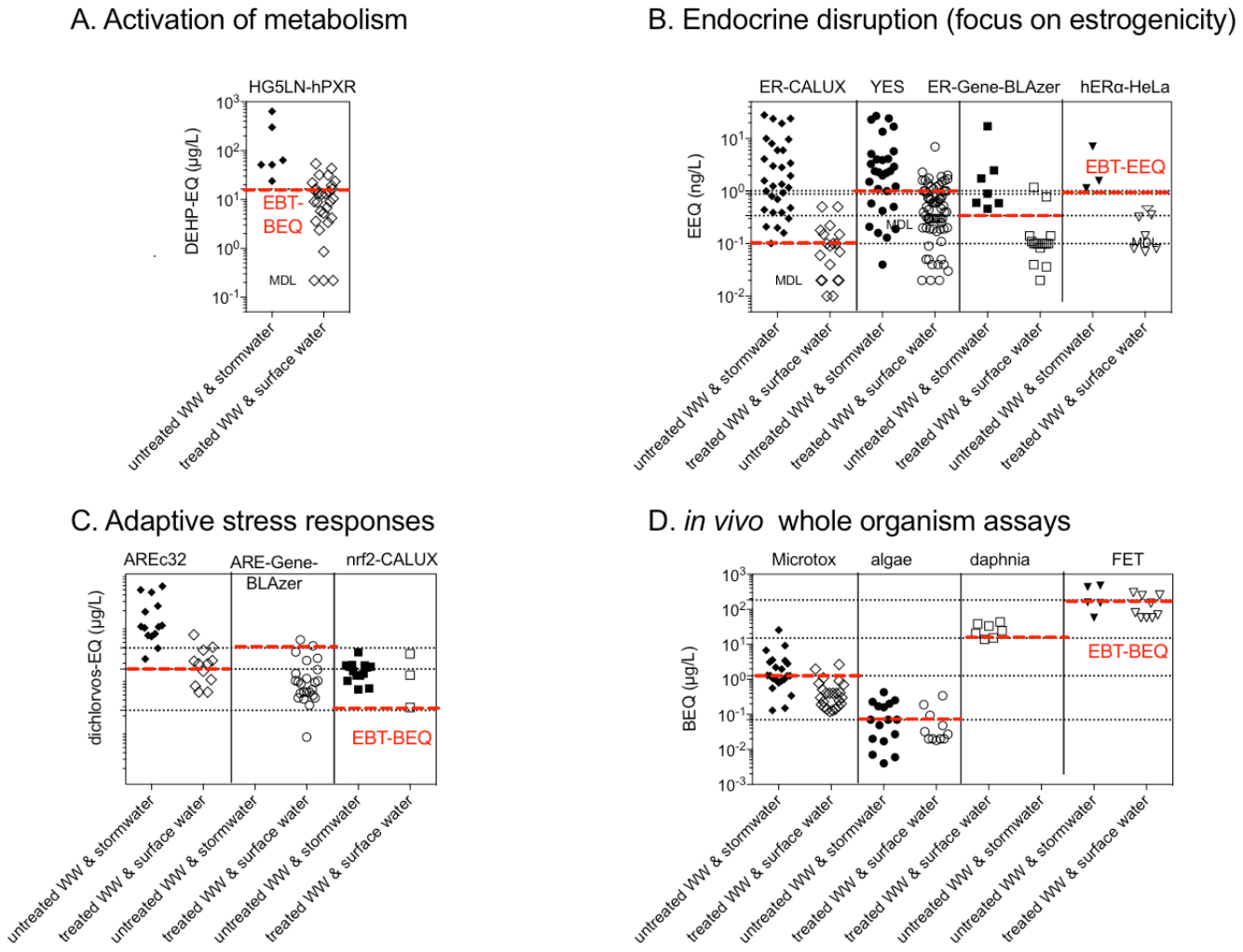


Fig 2. Comparison of the measured BE in field studies with the proposed EBT-BEQ
(figures adapted from appendix B of Escher et al. 2018)

In general, the EBT-BEQ fell in the range of the effects observed in the SOLUTIONS field studies and in

many cases they proved capable of differentiating observed water qualities. In the HG5LN-hPXR assay as an example for the activation of metabolism, the BEQ for all untreated water samples fell above the EBT and many of the treated and surface water samples were below (Figure 2A), Figure 2B also demonstrates for the example of four different estrogenicity assays that EBTs have to defined specific for each assay as only these show the ability to discriminate sample qualities. The same is true for the example of oxidative stress response as one adaptive stress response pathway, where also each bioassay must be assigned an assay-specific EBT (Figure 2C). Finally, for organism bioassays the formulated EBTs also show a tendency to help discriminating samples (Figure 2D). Clearly, however, more experience needs to be gained with field studies applying organism tests on SPE extracts of water.

Despite the limitations of these preliminary numerical EBTs, there is no doubt that EBT are required if bioassays are to become stand-alone monitoring tools. The methods details are further provided and discussed in deliverable 12.2.

Ecological observations

Exposure to toxic chemicals can affect freshwater life, and impact the viability of sensitive species. This can lead, especially for exposure over longer times, to shifts in the composition of freshwater communities. Toxic pressure is, however, not the only possible reason for variations in the community composition. It was previously observed that non-chemcial factors such as hydrology or general water quality can have a strong impact on the species composition. Hence, a statistical method was adapted in the SOLUTIONS project and applied for the detection of chemical impacts on biota and their discrimination from other, non-chemical stressors. The variance partitioning technique (Borcard et al. 1992) used, achieved a quantification of the variability in community composition that correlates exclusively with stressor groups. Technically, the variation partitioning is based on redundancy analysis, and the respective calculation algorithms are implemented in e.g. the CANOCO 5 software (Ter Braak and Šmilauer, 2012) or in R (varpart in package vegan).

For the application of the variance partitioning method, in addition to chemical exposure data and monitoring data on abundances of species, all available information about other stressors such as general water quality, or hydro-morphology are necessary. Based on the information about pollutants and other factors, the variance partitioning method yields the variabilities explained by the different groups of factors and rankings of those. By a stepwise refinement of the explaining factors, the resolution of the method can be increased by ‘zooming’ into single factor groups (Rico et al., 2016). In this way, a comprehensive statistical analysis of the relative importance of habitat characteristics, hydromorphological alterations, general water quality parameters such as nutrients, temperature or pH values, and metals or organic chemicals may be achieved.

In addition to the correlative assignment of the variance, groups of chemicals (e.g. grouped by use class or mode of action) can be ranked and statistically tested concerning their impact on the composition of species or their properties across a number of sampling sites using Monte Carlo permutation testing of the parameters to identify their isolated explanatory power. The methodology can be used to check whether measured chemicals show a statistically significant influence on existing community composition data.

The method was applied for a data set from the 3rd Joint Danube Survey, where for 55 sampling sites spread along the whole Danube, information on the community composition of aquatic macroinvertebrates, the concentrations of about 300 organic pollutants and data on habitat characteristics, hydromorphology and general water quality parameters were available (Rico et al., 2016). In this study the toxic pressure exerted to benthic macroinvertebrates in the Danube River was evaluated. Several variation partitioning analyses steps were performed to evaluate the relative contribution of contaminants and other abiotic parameters (i.e. habitat characteristics, hydromorphological alterations, water quality parameters) to the structural and biological trait variation of the invertebrate community. The calculated toxic pressure showed little variation between sampling sites, which complicates the identification of

pollution-induced effects. Variation structure and trait composition of the invertebrate community were mainly explained by habitat and water quality parameters, whereas hydromorphological alterations play a less important role. Among the water quality parameters, physico-chemical parameters such as suspended solids, nutrients or dissolved oxygen explained a larger part of the variation in the invertebrate community as compared to metals or organic contaminants.

A second application example was based on monitoring data from Swiss freshwater ecosystems, and targeted the detection of chemical impacts on the community composition upstream and downstream of wastewater treatment plant effluents (Burdon et al, in preparation).

These application examples illustrate strengths and limitations of the methodology. It provides a way to extract additional information by pooling existing data from different sources into one dataset. The statistical technique is standardised and can routinely be applied. It quantifies the actual impact of toxic pressure while simultaneously considering other factors and can hence confirm or reject hypotheses about chemical impacts arising from chemical pollution as indicated through chemical or bioanalytical analysis. A major limitation lies in that the method is based on correlations rather that causative links, meaning that it is prone to detect false positives. In addition, the data from different sources need to be consistent in terms of sampling time and location, a precondition which is not always met for standard monitoring data. A further limitation is that it is nearly impossible to detect the impact of single chemicals, as long as the number of monitoring sites is smaller than the number of potential impact factors.

3.3 Cause-effect relationships

Exploring strategies for the establishment of causal relationships between multiple contamination and deleterious biological effects in SOLUTIONS work has been performed with regard to two biological outcomes. Firstly, we operationalised the water framework directive concept of biological quality elements by using effect-based methods and deconstructing biological effects observed for water samples using effect-directed analysis. Secondly, we synthesised different lines of evidence to explain observable biological effects on communities in the field, which we here call ecology-directed analysis.

Effect-directed analysis

Despite the presence of mixtures of multiple compounds in environmental media, theoretical considerations and experimental findings suggest that the overall risk to individual organisms or populations of a species may be driven by only a few mixture components (Altenburger et al. 2004). Thus, it is of promise to seek to identify chemicals that contribute significantly to observed effects and to establish the corresponding cause-effect relationships in order to focus potential management.

While in some cases well-known chemicals can explain observed biological responses (e.g., estrogenicity detected with in-vitro assays in surface water samples can be explained to a large extent from natural and synthetic steroid estrogens such as estrone, estradiol, and ethinyl-estradiol, (König et al. 2017), quite often routinely analysed chemicals cannot explain observed biological responses (e.g., Escher et al. 2013), which points to a mismatch between these assessment approaches. Efforts in the SOLUTIONS project aimed to better understand which chemicals contribute to effects and which fraction of effect is caused by unknown chemicals through joint efforts for the different lines of evidence, resulting in a tiered approach shown in Figure 3.

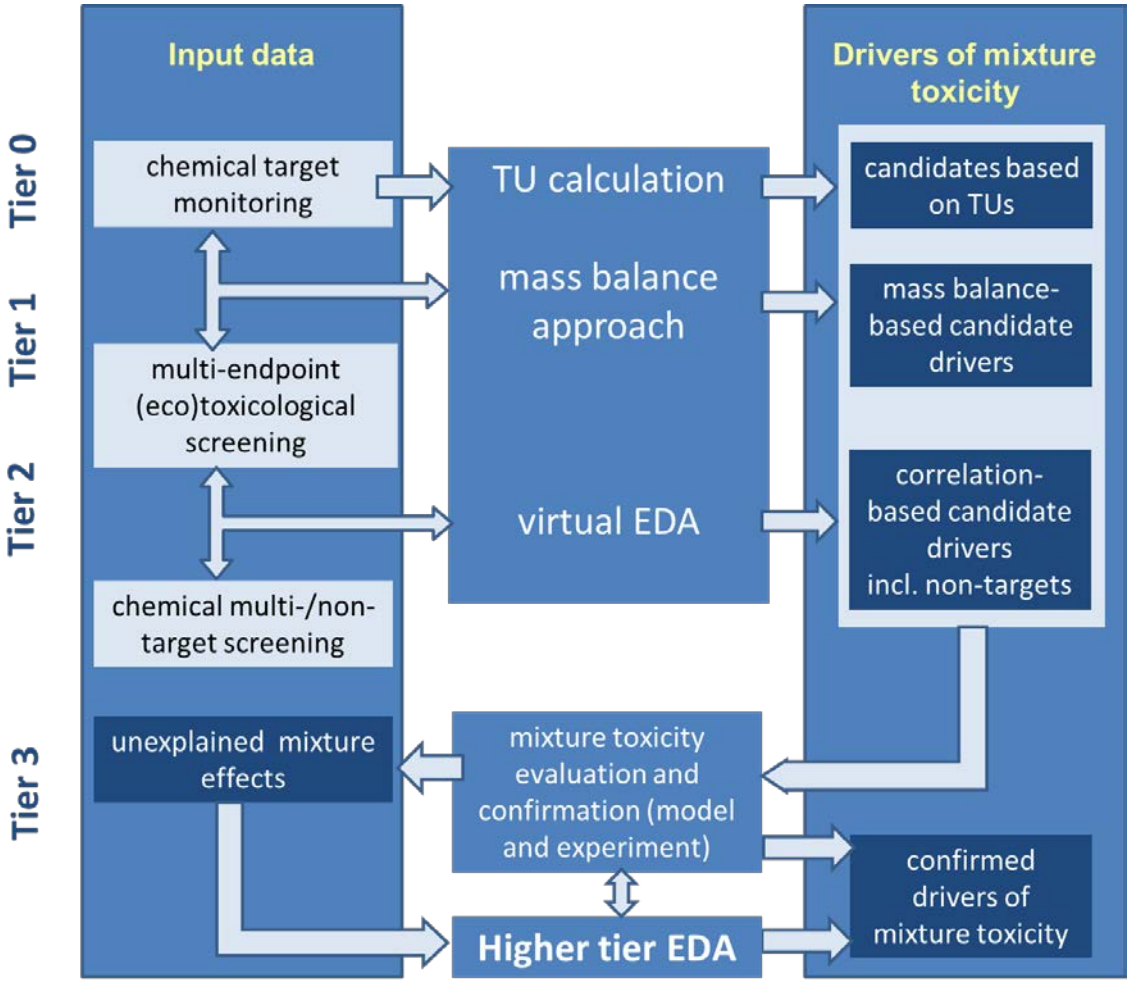


Figure 3: Framework for the tiered identification of cause-effect relationships *between chemical contaminants and selected biological effects illustrating the role of vEDA and HT-EDA in that framework. TU = toxic unit. Modified from Altenburger et al. (2015).*

When determining drivers of mixture toxicity, in tier 0 it is suggested to evaluate existing chemical target monitoring data only, i.e. if no effect monitoring is performed. Chemicals detected may be screened for modes of action and organisms at risk may be identified (Busch et al. 2016). For chemicals with known effect concentrations (ECs), Toxic Units (TUs) can be calculated as the ratio between environmental concentrations and ECs and used for the identification and prioritization of candidate drivers of toxicity and risk. Assuming concentration addition as a model for mixture effects, TUs can be summed up in a default mixture toxicity approach (Backhaus and Faust 2012). Tier 0 may provide a basis for a tentative prioritization of sites, rivers or organisms of concern, considering existing information. However, biological effects or risks may obviously be overlooked if chemical or toxicological data is incomplete.

In tier 1, existing chemical monitoring data may be complemented with (eco)toxicological screening using a set of relevant endpoints (Escher and Leusch 2012, Escher et al. 2014, Jálová et al. 2013) while chemical monitoring may be extended by target, suspect and non-target chemical (Hug et al. 2014, Krauss et al. 2010, Moschet et al. 2014) screening. The chemical and (eco)toxicological information can be integrated as for tier 0 or one can estimate how much of the measured effect(s) can be explained with the compounds detected in the chemical screening. This mass balance approach (Hilscherova et al. 2000) is based on the default mixture toxicity approach explained above and can be performed by comparing the sum of toxic units (∑Tus) or the sum of bioanalytical equivalent concentrations (∑BEQs) calculated using

individual chemicals concentrations from a chemical analysis with the toxicity of the sample in the bioassay, quantified as the required relative enrichment factor (REF) of a sample to achieve the endpoint of concern. More recent work has shown that it may not be reasonable to achieve a full mass balance for every biological endpoint. While for receptor-mediated effects a full mass balance is desirable, for nonspecific effects and adaptive stress responses the iceberg model described above may help to quantify the unknown effect, because for those endpoints, many if not all compounds occurring in a water sample may contribute to a mixture effect. It may therefore become a monitoring parameter in its own right.

At the next level (tier 2) in order to integrate chemical and (eco)toxicological information, a virtual EDA (vEDA) is suggested. This may serve to identify candidate chemicals which may explain the effects (Eide et al. 2004, Hug et al. 2015). The goal of a virtual EDA is to reduce the complexity of mixture components through the use of multivariate statistics and pattern recognition methods on samples from larger number of sites using a decomposition approach. This approach is able to handle peaks from non-target analysis and is not restricted to previously known chemicals. Virtual EDA results in peaks tat co-vary with observed toxicological effects, suggesting these as candidates representing causative chemicals. Obviously, this approach does not directly provide cause-effect relationships, but allows deriving hypotheses; which have to be confirmed using e.g. literature and database review, calculation of toxic units using QSARs, or a full chemical and effect assessment with reference standards. The application of vEDA requires that the following prerequisites are to be met:

1. The observed effect is caused by a limited (small) number of toxicants among all those present in a sample.
2. A successful application of vEDA requires some variance of the observed effect among the different samples. This should ideally be considerably larger than the uncertainty of the chemical and biological effect data.

Higher-tier EDA (HT-EDA, tier 3), to be used in the final step is a method that can be applied without any pre-information on types and sources of pollution. The data evaluation and investigations suggested in tier 1 and 2 will help to decide if a HT-EDA should be applied to unravel any remaining unexplained toxicity. Basically, HT-EDA reduce crude environmental sample extracts to less complex mixtures or individual compounds by bioassay-directed fractionation so that relevant toxicants can be isolated and identified by chemical analysis (Brack et al. 2016). Finally, identified toxicants need to be confirmed as the cause of the measured effect. This is carried out using analytical confirmation of identified structures, effect confirmation by testing neat standards and artificial mixtures thereof involving mixture toxicity modelling, and finally hazard confirmation, focusing on effects at higher levels of biological organization, such as populations and communities under realistic exposure condition.

As HT-EDA is a time- and resource-consuming approach, the problem formulation should be carried out with great care, prerequisites thoroughly checked and the methods and approaches selected appropriately. An application of HT-EDA is meaningful if

1. Effects can be observed in organic extracts of environmental samples (also implying being caused by organic chemicals).
2. The observed effects can be related to a specific toxicological endpoint, which can be assessed using bioassays applicable to environmental sample extracts and fractions within a reasonable time and cost scale.
3. The observed effect is caused by a limited (small) number of toxicants among all those present in a sample, which is discerned from the occurrence of a small number of active fractions in HT-EDA. This is mostly achieved for bioassays with specific, often receptor-mediated responses.

Ecology-directed analysis

For improving the causality between chemical occurrence and biologically adverse effects observable in the aquatic environment, we developed and explored a multiple lines of evidence approach thus assessing the plausibility of chemical-induced ecological effects. In complement to the above laid out tiered approach for the identification of cause-effect relationships for defined bioassay responses, this approach provides an adaptive and integrative method by synthesising different kinds of information. The assessment of the ecological quality for a certain site or a number of sites combines multiple lines of evidence in a weight of evidence approach. It is integrative; because it combines tests and tools from different levels of biological organisation (from cell tests to community data) with chemical exposure data in a schematic way (figure 4). It uses a statistically supported, transparent and formalized weight of evidence (WOE) approach with the aim to identify the biological quality of a certain site, in connection to the question whether chemicals have a possible impact. It is adaptive, because the approach can deal with missing lines of evidence. For the application of the weight of evidence approach, four main lines of evidence may be included (figure 3):

1. Predictive mixture modelling,
2. Effect-directed analysis (EDA),
3. *In situ* tests,
4. Field-based monitoring studies.

The suggested four lines of evidence have been integrated in a systematic and transparent WOE approach, based on a decision matrix. Further information on the approach is given in deliverable 13.1 (abstract see Annex E). For an application example, data obtained from the 3rd Joint Danube Survey for the different lines of evidence were analysed: Results from in depth chemical analyses of water samples were analysed by predictive mixture toxicity modelling (sum of toxic units, STU). Results from a suite of *in vitro* bioassays, performed with extracts from high volume and passive sampling, were taken into account as published (Schulze et al., 2015; Neale et al; 2015). Results from a battery of relevant in situ biomarkers in sentinel fish (Alburnus alburnus and Neogobius sp.) (Deutschmann et al., 2016) were analysed and aggregated using the average biomarker response. Finally, taxonomy- and traits-based analyses of fish and macroinvertebrate community data were performed to identify possible ecological impacts (Rico et al., 2016).

It was possible to transform the high-dimensional JDS3 data into a comprehensible matrix (figure 4), which summarise the overall evaluation without losing more precision than necessary. Now, this can serve as basis for conclusive statements about the impairment of the ecological status. The toolbox application resulted in the identification of a number of sites where all LOE indicate impairment, from predictive toxicity modelling over biomarker responses up to community level indicators. In total, the picture emerged that many of the Danube sampling sites show clear anthropogenic impacts, and in all of them the toxic pressure suggests toxicants can be considered a contributing cause. However, the biomarker responses (LOE3) for many sites indicate that the link from toxic pressure to community effects is not as clear as it might appear from only linking chemical pressure to community effects. Here, biomarkers and their aggregation in form of an average biomarker response show their potential to add a linking element to the overall evaluation of the chemical and ecological quality of water bodies.

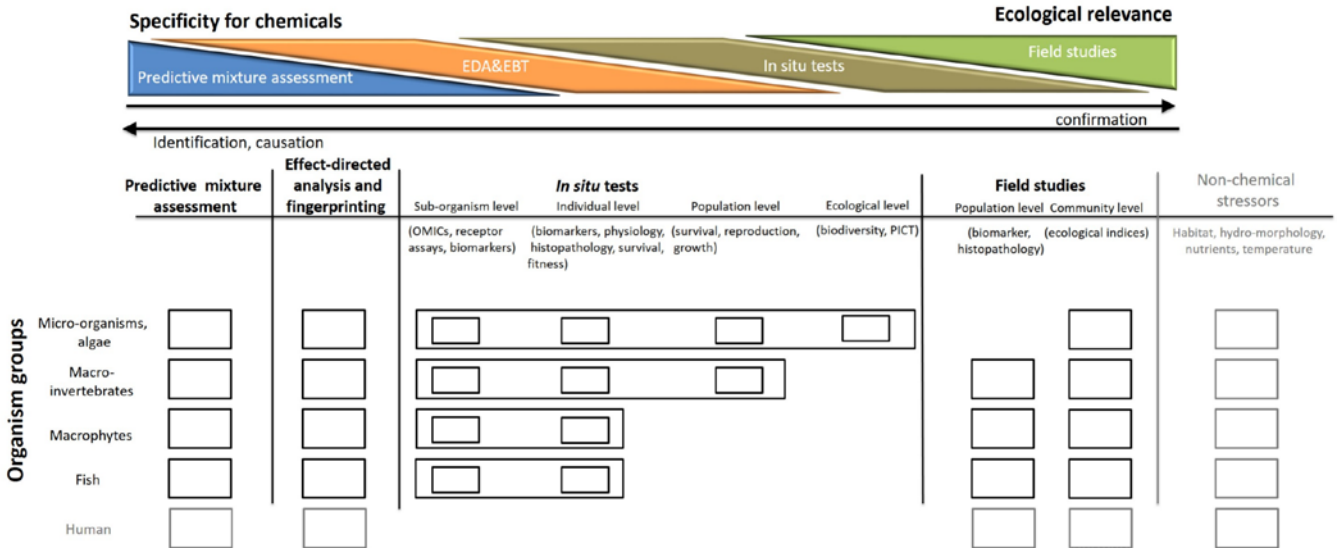


Figure 4: Aggregating information different lines of evidence into a weight of evidence (WOE) matrix *to support cause-effect relationships between chemical contamination and ecological effects; (top) Spectrum between chemistry (toxicity potential) and ecology (effects at community levels). In situ tests can have a linking function between those ends; (bottom) Four main lines of evidence, including the elements of evidence for which additional information may be included. The in situ LOE includes tests from sub-organism to the community level. Mechanistic information is conserved by differentiation between the lines of evidence for the different organisms groups. Interpretation of results for the main lines of evidence are exemplified in deliverable 13.1.(cf. Annex E)*

4. Uses of novel and advanced methods in water monitoring

The novel and advanced methods with regard to water sampling and preparation, chemical and bio-analytics that were provided through the SOLUTIONS research have been explored and tested in the three major case studies. Results of these efforts are documented and discussed in the respective deliverables for the individual case studies, namely for the Danube (deliverable 19.4), Rhine (deliverable 20.1), and Iberian Rivers (deliverable 21.1). Various publications on specific methods and applications are available through the SOLUTIONS website (https://www.solutions-project.eu/). Here, we summarize the experiences obtained in two directions: Firstly, the array of available techniques need to be scrutinised with regard to their specific utilities for particular issues of water quality, such as e.g. water abstraction for drinking water production, or the assessment of causes for ecological status deterioration for selecting adequate management options. Secondly, for water monitoring perspectives structured guidance on the different strategy options and steps in monitoring may be required. In the following we strive to illustrate the guiding principles.

4.1 Applicability of tools

Modern water monitoring pursues different purposes. Within the framework of the water directive the monitoring of the chemical status distinguishes between surveillance, operational, and investigative monitoring. The first two purposes currently relate to priority contaminants as legally defined, despite the knowledge that this in its majority covers legacy compounds only, although management options areoften lacking. However, at least cross-compliance with other regulation such as the sustainable use of plant protection products directive requires an extension of target analysis to cover active ingredients of pesticides of current use and adaptive sampling strategies to accommodate e.g. for an event-based monitoring of peak contamination patterns from diffuse agricultural sources. The definition of river basin specific pollutants as requested by the water framework directive also leads to advanced chemical analytical efforts in order to become capable of identifying relevant pollutants or impacts at larger scale and from a wider range of potential sources such waste water treatment. Management planning targeting specific source or sink remediation, or compound retention would yet call for another level of knowledge, namely the inclusion of biological effect information either with regard to biological quality elements, ecological status or ecosystem services. Moreover, there are closely related monitoring purposes such as the surveillance of effluents and status of receiving water bodies, or the safeguarding of water abstraction for agricultural use or drinking water production. Such purposes require e.g. fast and robust water quality assessments. Moreover, if effluent surveillance is connected with emission levies, the legal acceptance of a scaling tool becomes a major criterion. While chemical analytical techniques and ecological observations are established yet separately handled methods in water quality monitoring under the WFD this is less so for effect-based methods. The experiences documented here and aggregated in the second last lines of table 1 are therefore unique. It emerges that the SOLUTIONS consortium from its experiences strongly advocates an extension in the use of this methodology for various purposes in water quality monitoring. For the chemical analytical methodologies a major asset would be to adopt a sampling strategy, such as time integrating passive sampling that is matching the monitoring purpose.

In table 1 we provide a qualitative summary statement on the suitability of the different categories of tools explored within SOLUTIONS and tested in the different case studies. The summary is given in three categories ++ for highly suitable, + for suitable and – for less suitable. It has to be considered an expert statement, that remains to be qualified for the specificities of a given application purpose, the required assessment quality, the available resources and the specific tools considered. As a research consortium the group was charged to advance and explore chemical and biological tools for future water monitoring. The techniques explored range from fundamentally new, such as toxicogenomic techniques for untargeted effect analysis to robust and ring tested chemical analytical sampling and target analysis. The specific

state of the various tools and their use and performance in the three case studies (Danube, Rhine and Iberian rivers) are documented in the various reports and publications that can be accessed through the SOLUTIONS website (https://www.solutions-project.eu/). Beyond the scope of the team are considerations on aspects such as the legal validity of method-related results or the cost assessment. For the former again the legal frame and the specificities of member countries become most relevant as the WFD has to be adopted into national legislation. In Germany, for example, effect-based tools are well established and lawfully agreed in wastewater effluent monitoring and levy setting under the water law. Costs, on the other side may depend to a large extend on the availability of infrastructure and trained personnel and less so on the direct method-related incurred expenses.

Table 1: Matrix of applicability; suitability of sampling, chemical, effect-directed, bioanalytical and ecological tools for different water monitoring purposes

Methodology	**Surveillance and operational monitoring**	**Investigative monitoring**	**Identification of RBSP**	**Effluent surveillance**	**Water abstraction early warning**	**Identification of site-specifc toxicants**	**Impact assessment and Multiple stress diagnosis**
Adaptive sampling strategy	+	++	++	-	+	++	++
Target screening/ analysis	++	++	+	+	-	-	+
Suspect/non-target screening	-	+	++	-	+	+	+
Virtual EDA	-	+	++	+	-	-	+
Higher-Tier EDA	-	-	+	-	-	++	-
Effect-based analysis	++	+	+	++	++	++	+
Ecological analysis	-	++	++	-	-	+	++

4.2 Decision tree

As it was demonstrated in the chapter on the use of the various tools within various problem oriented strategies (chapter 3), typically a smart combination of methods is best to solve a specified water quality assessment problem. A seemingly simple question in water monitoring such as ‘how much variation in pesticide occurrence in small streams is due to rain events’ already requires an adequate monitoring strategy of event-based sampling and chemical target analysis to provide a reliable answer. It was also abstractly known and experienced through the interaction between stakeholders and scientists of the SOLUTIONS project that monitoring of water quality can be done for many specific objectives with varying demands. These different objectives drive the selection of monitoring tools. Typical demands comprise speediness, sensitivity, adequacy, statistical robustness, high reproducibility, accepted level of standardisation, automation protocol, demonstrated use for purpose, potential for inference, cost effectiveness and degree of representativeness. Clearly these demands may easily become mutually exclusive. Thus, recommendations on the use of specific techniques can only be derived in a context-dependent manner. In order to facilitate such a context building in a transparent and reproducible way SOLUTIONS developed and provides an expert decision tool, RiBaTox, for supporting a structured access to the various methods. The RiBaTox platform is accessible online at: https://solutions.marvin.vito.be/. Here, we provide the structure of the hierarchy guiding the user from general questions to specific monitoring tools and making the experiences accessible. Table 2 provides the underlying decision tree to help selecting specific sampling, chemical analytical, effect-based, toxicant identification and ecological tools for specific water monitoring strategies. The idea behind the stepwise decisions to be taken being that devising a monitoring strategy for a specific purpose requires the selection of methods out of several options. The central categories from which to choose elements are provisions on the sampling, the chemical analytical, the effect-based, the effect-directed or the ecology-directed methodologies. Among these the various options are laid out in detail in independent fact sheets, providing access to specific techniques, applications and documented case studies.

Table 2: Decision tree to help selecting specific sampling, chemical analytical, effect-based, toxicant identification and ecological tools for specific water monitoring strategies

Tree level 1	Tree level 2	Tree level 3	Fact sheet
Monitoring strategies			Strategies for monitoring of chemicals and their effects
	Sampling strategies		Sampling strategies
		Grab sampling	Grab sampling
		Passive sampling	Passive sampler for monitoring of trace organic contaminants in surface wate
		LVSPE	Large-volume solid phase extraction
		Event sampling	Event sampling
	Analytical strategies		Analytical strategies
		Target analysis	Protocols for target analysis of emerging contaminants in water, sediment ar
		SOPs compounds	Standard operational procedures (SOPs) for organic compounds
		SOPS compound classes	Standard operational procedures (SOPs) for compounds organic classes
		Preparation of standards	Syntheses of reference standards for SOLUTIONS
		Suspect screening	Screening for 'known unknown' or 'suspect' pollutants
		Non-target screening	Non-target screening and structure elucidation workflow
	Strategies for effect-based monitoring		Effect-Based Methods
		in vivo tools	*in vivo* tools
		in vitro tools	*in vitro* tools
		Benchmarks and trigger values	Benchmarks and trigger values
		Biological Early Warning Systems	Biological early warning systems (BEWS)
	Strategies for toxicant identification		Strategies for the identification of toxicity drivers
		Ecotoxicological mass balances	Ecotoxicological mass balances
		virtual EDA	Virtual EDA
		higher tier EDA	Higher Tier Effect-Directed Analysis
	Strategies for ecological assessment		Strategies for ecological assessment
		Macrofauna community based	Statistical approaches to discriminate multiple stressors
		PICT	Pollution-induced community tolerance for the in situ identification of ecolo
		Fish status assessment	Community and individual level markers for the identification of chemical ef
		Fish biomarkers	Biomarkers for exposure and effects of chemicals in fish
		Weight of evidence approaches	Weight of evidence approaches

5. Annexes: Supporting material

Annex A:
Abstract from deliverable D10.1
Guidelines for target and non-target analysis of emerging contaminants in water and biota

The number of analytical methods developed for targeted determination of emerging contaminants has experienced rapid growth in the last years and continues to increase with the discovery of new environmental contaminants and metabolites and transformation products (TPs). However, there are still significant challenges and gaps that need to be filled especially with regards to the availability of analytical methods for biota, the improvement of method detection limits for compounds with very low predicted no-effect concentrations (PNECs), and the detection, identification and structure elucidation of known and unknown components in complex environmental matrices with typically tens of thousands of components. In this context, the overall objective of the SOLUTIONS WP10 "Chemical Analytical Tools" was to develop novel methods for high-sensitivity detection and identification of new emerging environmental pollutants, metabolites and TPs, including a field-validated system-approach (passive sampling, innovative extraction and clean up, and novel analytical tools) for compounds suggested for prioritisation (see graphical abstract). To this end, different analytical approaches have been developed including (i) a comprehensive strategy for structural identification of emerging pollutants, their metabolites and TPs through to unknowns with the help of software tools for transformation product prediction as well as candidate selection, and (ii) diverse target analytical methods for detection and monitoring of numerous aquatic pollutants in water at levels below their PNECs, as well as in biota, based on the use of novel, advanced techniques in the various fields of sampling, sample preparation and analysis. Thus the present deliverable compiles in a systematic way the efforts made in each of these fields and the corresponding outcomes. Efforts made within the sampling field include the development of new passive sampling and large-volume in situ solid phase extraction (SPE) devices and methods, within the sample preparation field the testing of the high performance counter current chromatography (HPCCC) technique and the development of new methods based on automated on line SPE, and within non-target and target analysis field the design of a streamlined non-target screening workflow and a set of interacting compound identification tools, the synthesis of tentatively identified substances for identification confirmation, and the optimization of new analytical methods for high-sensitivity determination of target pollutants, metabolites and TPs in water and biota and their transformation into easy to follow standard operational procedures (SOPs).

Annex B:
Abstract from deliverable D11.1
SOP for mutually validated virtual and higher tier EDA of surface waters and fish tissue ready for translation into guidelines

Aquatic environments are often contaminated with complex mixtures of chemicals that may pose a risk to ecosystems and human health. This contamination cannot be addressed with target analysis alone; tools to reduce this complexity and identify those chemicals that might cause adverse effects are required. Effect-directed analysis (EDA) is designed to meet this challenge and faces increasing interest in water and biota quality monitoring. This deliverable summarizes current experience and provides guidance on the use of higher-tier EDA (HT-EDA) and virtual EDA (vEDA) to unravel the identity of compounds responsible for adverse effects exerted by complex mixtures. In classical, higher-tier EDA (HT-EDA), one or more fractionation steps are used to reduce the complexity of environmental mixtures to an extent that a limited number of chemical signals finally remain in an active fraction, from which an identification and confirmation of the causative compound(s) is possible. In contrast, virtual effect-directed analysis (vEDA) is an approach to reduce the complexity by 'virtual fractionation' applying multivariate statistical techniques on a larger set of environmental samples to correlate the chemical signals with observed effects. The deliverable highlights the need for proper problem formulation and gives general advice for

study design. As the HT-EDA approach is directed by toxicity, basic principles for the selection of bioassays are given. A specific focus is given to strategies for sampling and extraction since they strongly impact prioritization of toxicants in EDA. Reduction of sample complexity mainly relies on fractionation procedures, which are also discussed. Automated combinations of fractionation, biotesting and chemical analysis using so-called hyphenated tools can enhance the throughput and might reduce the risk of artefacts in laboratory work. The key to determining the chemical structures causing effects is analytical toxicant identification. The latest approaches, target-, suspect and non-target screening as well as unknown identification are discussed together with analytical and toxicological confirmation approaches. As application cases, three examples of HT-EDA for the identification of toxicants are presented (Sections 7-9), demonstrating the successful combination of the outlined approaches. The basic concept of lower-tier virtual EDA (vEDA) is to combine the existing or 'easily' to be obtained information derived from mass spectral analysis, bioanalysis and associated data, if available, by utilisation of multivariate statistics to isolate compounds or peaks (in case of non-target analysis) that co-vary with the responses or effects. Its role is to derive hypotheses on co-varying compounds that can be confirmed, e.g. by HT-EDA studies or single and mixture toxicity assessment. This deliverable discusses the general prerequisites of vEDA, establishes a theoretical concept and applies it in a proof-of-concept case (Section 10).

Annex C:
Abstract from deliverable D12.1
Improved Bioassay Solutions for Environmental Monitoring Based on Adverse Outcome Pathways

A central motivation of the SOLUTIONS project is to establish and provide advanced methods for environmental monitoring of freshwaters that are useful to identify contaminants of emerging concern in the context of the European Water Framework Directive. Recently it has become evident that contamination of European river basins with chemicals is not limited to a small number of priority compounds but rather contamination patterns are diverse and complex. This situation is not easily amenable by additional chemical analytical efforts. It has thus been suggested to complement chemical monitoring with effect-based tools which offers the scope required to capture groups of compounds as well as their transformation products and combined effects.

In this deliverable we report on the efforts within the SOLUTIONS project to improve the utility of bioassays for environmental monitoring. Improvements were sought with regard to provision of

i. Mode-of-action collation for compounds that are currently being detected in surface water monitoring studies in order to compare this with the capability of suggested effect-based tools to detect these contaminants;
ii. Uniform and transparent experimental procedures for the selected effect-based tools, that facilitate understanding of principle elements of their use and the data that they generate;
iii. Case studies from the Danube and Rhine river basins employing chemical and effect-based tools in concert to study the coherence and complementarity between chemical and bioanalytical information.

The next steps in the development of effect-based tools for water monitoring will include consideration and validation of mixture effects for the individual bioassays, verifying the relevance of combined effects in environmentally relevant mixtures, reflecting on the relationships between different bioanalytical tools and ecological effects, and developing guidelines for the use of effect-based tools for different monitoring purposes.

Annex D:
Abstract from deliverable D12.2

Feasibility assessment of extract-bioassay approaches for environmental monitoring

There is increasing interest in applying bioassays complementary to chemical analysis for water quality monitoring. While monitoring has traditionally applied in vivo methods, in vitro bioassays have advantages that support their application for testing water extracts, such as the ability to be run in high-throughput mode and low sample volume requirements. Further, they can provide information about potential acute and chronic effects for ecosystem health. In this deliverable we report on the work undertaken in SOLUTIONS, as well as complementary work by project partners, to assess the feasibility of extract-bioassays approaches for environmental monitoring. This includes:

(i) Establishing bioassay test batteries that are not only based on the adverse outcome pathway, but also take into account effects observed in surface waters.

(ii) Outlining quality considerations for bioassays to help ensure that bioassays applied for monitoring are sensitive, specific and selective.

(iii) Evaluating the suitability of methods applied for enrichment of water samples prior to bioanalysis by considering effect recovery and blank effects.

(iv) Developing effect-based trigger values for in vitro bioassays to distinguish between acceptable and unacceptable water quality.

The proposed modular batteries include whole-organism and cell-based bioassays but legally all are in vitro assays. The test batteries have been applied in SOLUTIONS case studies.

This work conducted as part of SOLUTIONS and in collaboration with the NORMAN network represents a major advance in the current understanding regarding the suitability of extraction/bioassay approaches for water quality monitoring and provides further support for their application.

Annex E:
Abstract from deliverable D13.1
Diagnostic toolbox for ecological effects of pollutant mixtures, including bio-tests, trait-based database and detection tool and WoE studies at hot-spot sites

The toolbox for the detection of the ecological impact of chemicals uses a statistically supported, transparent and formalized weight of evidence (WOE) approach that integrates four main individual lines of evidence (LOEs), (i) predictive mixture modelling, (ii) effect-directed analysis (EDA), (iii) in situ tests, and (iv) field-based monitoring studies. A systematic and quantitative method was developed for the aggregation of multiple in situ test results into one LOE, resulting in the definition of the average biomarker response (ABR). Integration of single LOE in a weight of evidence approach was defined in form of a decision matrix. The main idea of the approach is to systematically integrate these four LOEs, so that their strengths complement each other and allow a transparent site-specific assessment with particular attention to the establishment of links between chemical exposure and ecological impacts, identification of data gaps and management options. The focus for the development was to keep the methodology simple enough to enable routine use by non-scientists. Three practical weight of evidence examples are presented in addition, illustrating specific aspects of weight of evidence studies. The developed toolbox was applied to the Danube case study, to facilitate evaluation of the very comprehensive data set from Joint Danube Survey 3. The Rhine and the Holtemme cases are smaller scale studies focused on site specific toolbox application in an upstream/downstream set-up. The toolbox concept proved to be practical, simple and promising for further studies, with fairly high diagnostic power.

Annex F:
Summary of SOLUTIONS studies on

Non-targeted bioanalytical methods for water monitoring

To pursue the objective of exploring in how far non-targeted bioanalytical methods may complement existing cell- and organism-based methods, we focussed on transcriptomic signals following short-term exposures. We investigated bacterial, daphnid and fish biosystems to reflect the different biological quality elements of pelagic systems and to capture the relation between biosystem-specific and unspecific chemical activity. Thereby, we applied toxicogenomic approaches with two major goals: a) to gain a deeper understanding of molecular processes as response to chemical stress and mixture exposures and b) to investigate the sensitivity and specificity of toxicogenomics for diagnostic purposes.

We employed different systems for effect-detection: off-target transcript detection to observe for immune system responses in primary fish cells (UOB); a reduced transcriptome approach (NJU); and whole genome microarray studies (UB, UL, UFZ). Furthermore, we performed top-down investigations on water samples during the JDS3 as well as on specific case studies (e.g. the Danube in Novisad - at the waste water sewage site, as well as upstream and downstream). Bottom-up studies were performed with individual water pollutants and their mixtures in different experimental designs addressing questions on specificity and sensitivity of the omics approaches.

Major findings and conclusions drawn from the different studies are summarised below ordered according to the different systems used and questions raised.

1. Transcriptome studies using zebrafish embryo systems
2. Transcriptome studies using water flea systems
3. Transcriptome studies using bacteria systems
4. Transcriptome studies using primary rainbow trout fish cells

Ad 1: Transcriptome studies using zebrafish embryo systems (UFZ & NJU)

In this part we worked to establish a workflow for a component-based combined response prediction and assessment for mixture exposures on a transcriptome level.

As a first step to pursue the ambition, good statistical analysis of toxicogenomic data is vital. In contrast to established ecotoxicological techniques, concentration-response modelling is rarely used for large datasets. Instead statistical hypothesis testing is prevalent, which provides only limited scope for inference. This first study therefore applied automated concentration-response modelling for three different ecotoxico-transcriptomic and ecotoxico-metabolomic datasets. The modelling process was performed by simultaneously applying nine different regression models, representing distinct mechanistic, toxicological and statistical ideas that result in different curve shapes. The best fitting models were selected using Akaike's information criterion. The linear and exponential models represented the best data description for more than 50 % of responses. Models generating U-shaped curves were frequently selected for transcriptomic signals (30 %), and sigmoid models were identified as best fit for many metabolomic signals (21 %). Thus, selecting the models from an array of different types seems appropriate, since concentration-response functions may vary due to the observed response type and also depend on the compound, the organism, and the investigated concentration and exposure duration range. The application of concentration-response models can help to further tap the potential of omics data and

is a necessary step for quantitative mixture effect assessment at the molecular response level. The method and experiences were published in Smetanová et al. 2015.

In a second step, we intended to identify and interpret genes and gene sets, which constitute a general response to chemical exposure, by reanalyzing published toxicogenomics data obtained with the ZFE. Additionally, we aimed at identifying factors responsible for the extent of gene regulation in an experiment. We identified differentially expressed genes (DEGs) for each treatment using a moderated t-test and subsequently analyzed the overlap of DEGs between the treatments as well as the proportion of regulated genes among all tested genes. Next, we conducted an effect size analysis across all treatments and subgroups. Using permutation analysis we identified commonly regulated "meta-genes". Overlap of significantly regulated genes between independent studies was generally low (<50%). However, effect size analysis revealed several genes showing a common trend of regulation among which genes related to calcium homeostasis emerged as key, especially for early exposures. Also, these and other downregulated genes are often linked to anatomical regions developing during the respective exposure period. These efforts were published in Schüttler et al. 2017.

In a third step we strived to understand the role of exposure time point in zebrafish embryos for the induction of transcriptional changes. Especially transcripts that code for biotransformation proteins play essential roles in the temporal development of toxicity or in detoxification processes. However, we hypothesised that such an active and directed response is not available in early zebrafish embryo stages. The time windows during development where such responses take place and are detectable is important for experimental designs and the interpretation of omics data. We, therefore studied the biotransformation capabilities of two different stages of zebrafish embryos by exposing zebrafish embryos of the late cleavage/early blastula period (2-26 hpf) and the early pharyngula period (26-50 hpf) for 24 h, respectively, to the AhR binding compound benz[a]anthracene (BaA). The time dependent changes in cyp gene transcription for cyp1a, cyp1b1, cyp1c1 and cyp1c2, as well as concentration and time-dependent courses of BaA concentrations in the fish embryo and the exposure medium were analysed in a targeted approach. Additionally, the CYP mediated formation of biotransformation products was investigated. We found correlations between transcriptional responses and the internal concentration for both exposure types. These correlations were depending on the start of the exposure i.e. the age of the exposed embryo. While no significant induction of the examined gene transcripts was observed in the first 12 h of exposure beginning in the blastula period a correlation was apparent when exposure started later i.e. in the pharyngula period. A significant induction of cyp1a was detected already after 1.5 h of BaA exposure. Gene transcripts for cyp1b1, cyp1c1 and cyp1c2 showed expressions distinctly different from cyp1a and were, in general, less inducible by BaA in both exposure windows. The toxicokinetic analysis showed that the biotransformation capability was fivefold higher in the older fish embryos. Biotransformation products of phase I reactions were found between 32 hpf and 50 hpf and were tentatively identified as benz[a]anthracene-phenol and benz[a]anthracene-dihydrodiol-epoxide. In conclusion, not only duration but also onset of exposure in relation to the developmental stage of zebrafish embryos is important in the analysis and interpretation of effects and toxicity pathways due to different biotransformation capabilities. These findings are published in Kühnert et al. 2017.

Finally, we studied three water contaminants (Diuron, Diclofenac and Naproxen) individually and in mixture using a zebrafish microarray in a time and concentration-resolved experiment considering the lessons learned from earlier studies with respect to exposure time points and concentrations. Using an

advanced experimental design, we investigated the detectability and predictability of combined effects using extended component-based mixture modelling. The experimental work is completed, while the data analysis is still ongoing (Schüttler et al. in prep.).

Having understood the role of exposure time points and durations as well as the sensitivity of the transcriptome we can start to reduce the whole genome towards those pathways that are toxicologically relevant for high throughput applications. Therefore, in a next step, a reduced zebrafish transcriptome (RZT) approach was developed to represent the whole transcriptome and to profile bioactivity of chemical and environmental mixtures in zebrafish embryo. A RZT gene set of 1637 zebrafish genes was designed to cover a wide range of biological processes and to faithfully capture gene-level and pathway level changes by toxicants compared with the whole transcriptome. Concentration−response modelling based on the approach by Smetanová et al. (2015) was used to calculate the effect concentrations of differentially expressed genes and corresponding molecular pathways. To validate the RZT approach, quantitative analysis of gene expression by RNA-ampliseq technology was used to identify differentially expressed genes at 32 hpf following exposure to seven serial dilutions of reference chemical BPA or each of four water samples ranging from wastewater to drinking water. The RZT-ampliseq-embryo approach was both sensitive and able to identify a wide spectrum of biological activities associated with BPA exposure. These findings were published in Wang et al. 2018.

Conclusions from 1.

A distinct general stress response that is invariant to the type of chemical exposure could not be identified in the currently available zebrafish transcriptome studies with chemical exposure. In subgroups, however, potential key events of toxicity could be discriminated. Improved time- and concentration-resolved experiments offer access to a more detailed mechanistic understanding to inform risk assessors and enable experimental designs based on hypothesis. The developed reduced zebrafish transcriptome approach provides a sensitive diagnostic tool for the assessment of the total molecular effect caused by a chemical or sample. So far, the observations available remain inconclusive whether observed multiple responses in transcriptomic patterns upon mixture exposure can be used to identify or discriminate the occurrence of a specific mode of action against a background of noise and overlapping signals from other influencing factors.

Ad 2: Transcriptome studies using water flea systems (UB & UL)

Using the water flea (*Daphnia magna*) we aimed at comparing top down and bottom up investigations with respect to sensitivity and response overlaps. Therefore, *Daphnia magna* transcriptional profiles were determined after exposure to complex environmental large-volume solid phase extracted (LV-SPE) surface water samples from the Danube River, the acetylcholinesterase (AChE) inhibitor insecticide diazinon and the herbicide diuron, a binary mixture of these two compounds and an artificial multi-component mixture also containing diazinon and diuron. The single compound exposures to diazinon and diuron, as well as the binary and artificial multi-component mixture exposures all elicited significant transcriptional changes in Daphnia. Specific transcriptional signatures for the two model compounds were found which were detected within responses to the binary exposure as well as within responses to an artificial multi-component mixture. The Danube extracts segregated geographically using hierarchical clustering of the transcriptomic responses the samples from different sites elicited in Daphnia. Toxicologically important transcripts (e.g. CYPs, GSTs, SOD) were significantly induced by extracts

from Novi-Sad, the middle and lower Danube, but not the upper Danube. There was a significant similarity ($P<0.012$) between archived AChE-inhibitor response data and Daphnia responses to the lower Danube extracts. Finally, single compound, binary and mixture transcriptomic signatures could be detected within responses to the complex environmental LV-SPE samples from the Danube. Daphnia transcriptomic studies therefore identified key toxicant classes within the context of an artificial mixture or complex environmental samples in a top down approach. The bottom up approach with single compound exposures additionally helped to verify and extend the list of key responding genes related to the pathway of AChE inhibition in Daphnia. The data analyses and final interpretation is still in progress (Williams et al. in prep.).

Conclusions from 2.

Untargeted molecular responses in Daphnia from a top down approach reflect the exposure situations and can partly be linked to known pathways in cases where prior knowledge is available. The pending data analysis of the bottom up mixture exposures will show to which extent the molecular responses reflect the single compound pathways and interactions between them. Finally, the sensitivity of the Daphnia transcriptome in terms of total transcriptional changes in comparison to other endpoints needs to be clarified.

Ad 3: Transcriptome studies using bacteria systems (NJU)

Functional genome-wide knockout mutants screening approaches are applied to identify specific chemical-gene interactions for compounds with unknown molecular biological action. Here *Escherichia coli* (E. coli) genome-wide knockout screening was performed in order to compare narcotic and specific acting chemicals. Therefore, three narcotic chemicals (4-chlorophenol (4-CP), 3, 4-dichloroaniline (DCA) and 2, 2, 2- trichloroethanol (TCE)) and three specifically acting chemicals (triclosan (TCS), clarithromycin (CLARY), sulfamethoxazole (SMX)) were investigated. 66, 97, 88, 144, 198 and 180 initial robust hits were identified by exposure to 4-CP, DCA, TCE, TCS, CLARY and SMX with two replicates at the concentration of the individual IC50, respectively. The average fold change values of responsive mutants to the three narcotic chemicals were smaller than those for the three specifically acting chemicals. The common gene ontology (GO) term of biological process enriched by the three narcotic chemicals was “response to external stimulus” (GO: 0009605). Other GO terms like “lipopolysaccharide biosynthetic process” (induced by 4-CP) and “purine nucleotide biosynthetic process” (induced by DCA) were also influenced by the narcotic chemicals. Four genes (flhC, fliN, fliH and flhD) were identified as potential biomarkers to distinguish narcotic chemicals and specifically acting chemicals. Furthermore, this assay was applied to investigate a brominated flame retardant with widespread production but very limited toxicological data, namely 2,2-bis(bromomethyl)-1,3-propanediol (BMP). 119 initial, including 66 sensitive and 53 resistant single gene mutants, were identified by a full library screening of BMP at the concentration of its IC50. The resistant genes were significantly enriched in nucleobase-containing compound biosynthetic process (GO: 0034654) by gene ontology (GO) biological process analyses, which suggested that the pathway of DNA repair is a critical cellular process in the survival of cells exposed to BMP. Meanwhile, functional annotation of all BMP responsive genes suggested the mechanism of BMP was associated with DNA damage, oxidative stress and cellular transmembrane transport process. Many genes were exclusively responsive to BMP compared with the other chemicals that have been assessed with the *E. coli* mutant screening approach, which indicated that

BMP has a distinct mode of toxic action. These findings are published in Guan et al. 2017 and Guan et al. 2018.

Conclusions from 3.

The *E. coli* functional genomic approach seems to be suitable to distinguish chemicals of different classes and provide information on gene interaction networks in *E.coli*. However, in order to determine the suitability for the assessment of environmentally relevant compounds a larger screening approach with a larger set of compounds is necessary. Nevertheless, we assume that a distinction of chemical polluted water samples from less polluted ones should be possible using this assay. Therefore, it provides potential for effect based monitoring which needs to be investigated and verified in future studies.

Ad 4: Transcriptome studies using primary rainbow trout fish cells (UOB)

Chemical influence on the immune system of fish is anticipated to contribute to chronic adverse effects. However, the knowledge on structures and functions of immune system and responses to exposure in fish species is limited and its role in the development of toxicity is not clear. Rehberger and colleagues, therefore, undertook to review and summarize what is known about immunotoxicology in fish by looking and analysing 241 scientific studies that investigated many different environmentally relevant chemicals and studied the responses of immune system related parameters, such as cell numbers and behaviour of specific cell types or targeted expression of specific genes (Rehberger et al. 2017). In a follow up study, it was investigated whether a distinction between immunotoxic chemicals and non-immunotoxic chemicals is possible by selecting endpoints and parameters for the detection of immunotoxicity based on the review results. Among the selected immune parameters were the cytokine genes IL-1β, TNFα, and IL-10. The expression of those genes should be a marker for immunotoxicity. Our hypothesis was that non-cytotoxic concentrations of compounds with known immunotoxic MoA in fish would specifically impact the immune functional parameters of the trout leukocytes, whereas compounds with narcotic (non-immunotoxic) MoA would cause no effects. This hypothesis could not be confirmed as non-immunotoxic compounds also caused significant effects on the selected endpoints.

Conclusions from 4.

Several targeted gene expression studies were found in literature for the detection of immunotoxicity in fish. However, these studies as well as our own study show that cytokine gene expression in fish immune cells seems not to be specific for an immunotoxic MoA. An untargeted omics approach focussing on immunotoxicity has not been performed yet, also because of the still patchy annotations of genomes of many fish species. However, our studies show that addressing the immunotoxicity of environmental chemicals requires a broader approach looking at more immune system related genes and pathways. Techniques, such as whole transcriptome sequencing, could serve this and should be considered in future studies on the immunotoxicity of environmentally relevant chemicals.

Summary and outlook

Targeted and untargeted omics approaches were applied within the SOLUTIONS project mainly in order to detect changes on the transcriptome of different species to answer diverse questions related to the understanding of molecular pathways of chemical toxicity with focus on mixture toxicity, neurotoxicity,

and immunotoxicity on the one hand and on the development of tools and approaches for handling and analysing large data and samples sets.

The toxicogenomics data analysed in this project were either generated in large, carefully designed experiments or retrieved from databases in order to address different research questions. Using and analysing the responses of whole transcriptomes to single compound exposure led to the identification of compound specific patterns (Wang et al. 2018, Guan et al. 2017 & 2018, Schüttler et al. in prep.) as well as more general responses and limitations of experimental approaches (Schüttler et al. 2017). The development of automated dose response modelling for omics data enabled a more process based understanding of transcriptomic responses and provides a starting point for mixture predictions and experimental designs that consider the dynamics of changes of gene expression after chemical exposure dependent on exposure concentrations (Smetanová et al. 2015).

The review article of Zhang et al. (2018) summarised the techniques established in the ecotoxicogenomics field by the Chinese SOLUTIONS project partner (NJU). Next to the above discussed approaches, also the application of omics techniques for eDNA metabarcoding of environmental communities is discussed in this review article. This is in interesting approach which was not addressed within the SOLUTIONS project but opens another field to become diagnostic with respect to linking chemical exposure and ecological effects.

Changes in the expression of different genes and pathways are thought to serve as proxies for modes of action as well as for long term adverse outcome. Here we found, within the different studies performed with either single compounds, mixtures or environmental samples, that gene expression changes can be observed in concentration ranges that relate to the lower part of a mortality dose response curve whereas concentrations that are more than 10 to 100 times lower as lowest mortality causing concentrations are not likely to cause effects on the gene expression level in short term exposures. Also, the identification of single responding genes and pathways was possible which either could be explained by components of a complex mixture using prior knowledge data analysis approaches (top down; Williams et al. in prep.) or indicate compound specific patterns that can be used in diagnostic approaches and for AOP developments (bottom up, Williams et al. in prep., Schüttler et al. in prep, Guan et al. 2017 & 2018, Kühnert et al. 2017). Identified key signals could now be used for the development of reduced diagnostic tools, such as the reduced zebrafish transcriptome approach by Wang et al. (2018) or need to be linked to adverse outcome pathways and toxic modes of action. As toxicity on the apical level is dose and time dependent also responses on the gene expression level can indicate adaptation or adversity. With the here designed experiments, which consider these time and dose aspects, it will become possible to distinguish adaptive and adverse pathways. However, the huge amount of data, gained in this project, need further exploration in order to anchor key genes and pathways with AOPs and MoAs in subsequent studies. Figure 5 summarises the activities and achievements of WP12 in toxicogenomics.

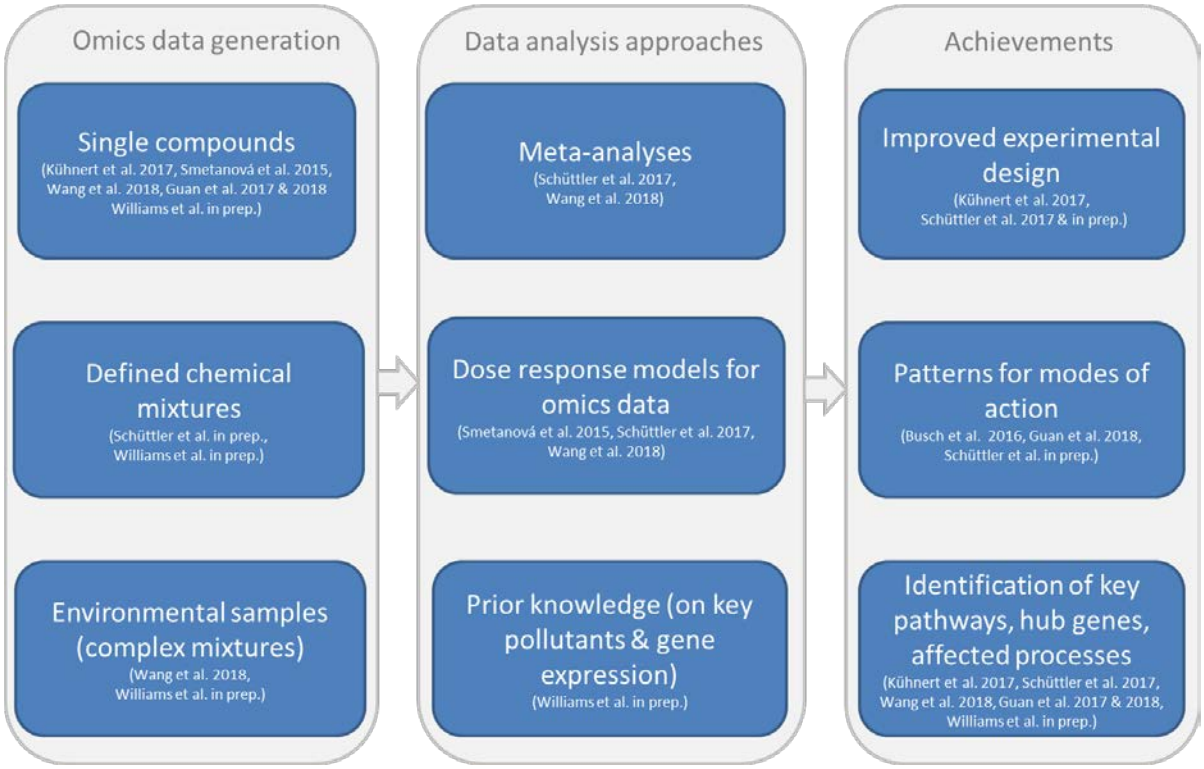


Fig. 5: Summary of different steps, approaches and achievements in the field of toxicogenomics *with respect to environmental monitoring and risk assessment*

6. Conclusions

From the development, exploration and use of various experimental and observational tools for water monitoring in the SOLUTIONS project and its case studies, suggestions can be provided for the advancement of future water monitoring under the provision of the water framework directive and the like. In brief these comprise:

- Efficient water quality monitoring requires specification of purpose and suitable composition of chemical and bioanalytical monitoring tools for the design of targeted monitoring strategies;
- Effect-based methods are of demonstrated complementary values for both, support of pollutant identification, as well as support of causality establishment between occurrence of chemicals and deleterious biological outcome;
- Sampling and chemical analytics can be based on advanced technique to more comprehensively account for contamination of waters and to identify sources;
- Extended monitoring efforts pay off in terms of a more holistic assessment of biological impacts of water contaminants, better understanding of drivers of mixture effects, and identification of priority mixtures.

7. References

Altenburger, R., Ait-Aissa, S., Antczak, P., Backhaus, T., Barcelo, D., Seiler, T.-B., Brion, F., Busch, W., Chipman, K., Lopez de Alda, M., de Aragao Umbuzeiro, G., Escher, B.I., Falciani, F., Faust, M., Focks, A., Hilscherova, K., Hollender, J., Hollert, H., Jaeger, F., Jahnke, A., Kortenkamp, A., Krauss, M., Lemkine, G.F., Munthe, J., Neumann, S., Schymanski, E.L., Scrimshaw, M., Segner, H., Slobodnik, J., Smedes, F., Kughathas, S., Teodorovic, I., Tindall, A.J., Tollefsen, K.E., Walz, K.-H., Williams, T.D., Van den Brink, P.J., van Gils, J., Vrana, B., Zhang, X. and Brack, W. (2015) Future water quality monitoring - Adapting tools to deal with mixtures of pollutants in water resource management. Science of The Total Environment 512, 540-551.

Altenburger, R., Scholze, M., Busch, W., Escher, B.I., Jakobs, G., Krauss, M., Krüger, J., Neale, P.A., Aït-Aïssa, S., Almeida, A.C., Seiler, T.-B., Brion, F., Hilscherová, K., Hollert, H., Novák, J., Schlichting, R., Serra, H., Shao, Y., Tindall, A.J., Tolefsen, K.E., de Aragão Umbuzeiro, G., Williams, T.D. and Kortenkamp, A. (2018). Mixture effects in samples of multiple contaminants – an inter-laboratory study with manifold bioassays. Environment International, 114: 95-116.

Altenburger, R., Walter, H., Grote, M., (2004): What contributes to the combined effect of a complex mixture? Environ. Sci. Technol. 38 (23), 6353 - 6362

Backhaus T., Faust, M. 2012. Predictive anvironmental risk assessment of chemical mixtures: aconceptual framework. Environ Sci Technol 46: 2564-2573.

Backhaus T, Segner H, Hollert H, Deutschmann B, Van den Brink P, Seiler T, Teodorovic I, Focks A. (2017) Solutions External Deliverable 13.1: Diagnostic toolbox for ecological effects of pollutant mixtures

Borcard, D., P. Legendre & P. Drapeau. 1992. Partialling out the spatial component of ecological variation. Ecology 73: 1045–1055.

Brack, W., Ait-Aissa, S., Burgess, R.M., Busch, W., Creusot, N., Di Paolo, C., Escher, B.I., Mark Hewitt, L., Hilscherova, K., Hollender, J., Hollert, H., Jonker, W., Kool, J., Lamoree, M., Muschket, M., Neumann, S., Rostkowski, P., Ruttkies, C., Schollee, J., Schymanski, E.L., Schulze, T., Seiler, T.-B., Tindall, A.J., De Aragão Umbuzeiro, G., Vrana, B. and Krauss, M. (2016) Effect-directed analysis supporting monitoring of aquatic environments — An in-depth overview. Science of The Total Environment 544, 1073-1118.

Brack, W., Dulio, V., Agerstrand, M., Allan, I., Altenburger, R., Brinkmann, M., Bunke, D., Burgess, R.M., Cousins, I., Escher, B.I., Hernandez, F.J., Hewitt, L.M., Hilscherova, K., Hollender, J., Hollert, H., Kase, R., Klauer, B., Lindim, C., Herraez, D.L., Miege, C., Munthe, J., O'Toole, S., Posthuma, L., Rudel, H., Schafer, R.B., Sengl, M., Smedes, F., van de Meent, D., van den Brink, P.J., van Gils, J., van Wezel, A.P., Vethaak, A.D., Vermeirssen, E., von der Ohe, P.C. and Vrana, B. (2017). Towards the review of the European Union Water Framework Directive: Recommendations for more efficient assessment and management of chemical contamination in European surface water resources. Science of the Total Environment, 576: 720-737.

Busch, W., Schmidt, S., Kühne, R., Schulze, T., Krauss, M. and Altenburger, R. (2016) Micropollutants in European rivers: A mode of action survey to support the development of effect-based tools for water monitoring. Environmental Toxicology and Chemistry, 1887-1898.

Deutschmann, B., Kolarevic, S., Brack, W., Kaisarevic, S., Kostic, J., Kracun-Kolarevic, M., Liska, I., Paunovic, M., Seiler, TB., Shao, Y., Sipos, S., Slobodnik, J., Teodorovic, I., Vukovic-Gacic, B and, Hollert, H. (2016). Longitudinal profiles of the genotoxic potential of the River Danube on erythrocytes of wild fish assessed using the comet and micronucleus assay. Science of the Total Environment, 573, 1441-1449

Eide, I., Neverdal, G., Thorvaldsen, B., Arneberg, R., Grung, B.r. and Kvalheim, O.M. (2004) Toxicological evaluation of complex mixtures: fingerprinting and multivariate analysis. Environmental Toxicology and Pharmacology 18(2), 127-133.

Escher, B. and Leusch, F. (2012) Bioanalytical tools in water quality assessment, IWA Publishing, London, UK.

Escher, B.I., van Daele, C., Dutt, M., Tang, J.Y.M. and Altenburger, R. (2013). Most oxidative stress

response in water samples comes from unknown chemicals: the need for effect-based water quality trigger values. Environmental Science & Technology, 47(13): 7002-7011.

Escher, B.I., Allinson, M., Altenburger, R., Bain, P.A., Balaguer, P., Busch, W., Crago, J., Denslow, N.D., Dopp, E., Hilscherova, K., Humpage, A.R., Kumar, A., Grimaldi, M., Jayasinghe, B.S., Jarosova, B., Jia, A., Makarov, S., Maruya, K.A., Medvedev, A., Mehinto, A.C., Mendez, J.E., Poulsen, A., Prochazka, E., Richard, J., Schifferli, A., Schlenk, D., Scholz, S., Shiraishi, F., Snyder, S., Su, G., Tang, J.Y.M., Burg, B.v.d., Linden, S.C.v.d., Werner, I., Westerheide, S.D., Wong, C.K.C., Yang, M., Yeung, B.H.Y., Zhang, X. and Leusch, F.D.L. (2014) Benchmarking organic micropollutants in wastewater, recycled water and drinking water with *in vitro* bioassays. Environmental Science & Technology 48(3), 1940-1956.

Escher, B.I., Ait-Aissa, S., Behnisch, P.A., Brack, W., Brion, F., Brouwer, A., Buchinger, S., Crawford, S., Hamers, T.H.M., Hettwer, K., Hilscherova, K., Hollert, H., Kase, R., Kienle, C., Legradi, J., Tuerk, J., van der Oost, R., Vermeirssen, E. and Neale, P.A. (2018). Effect-based trigger values for in vitro and in vivo bioassays performed on surface water extracts supporting the environmental quality standards (EQS) of the European Water Framework Directive. Science of the Total Environment, 628-629: 748-765.

Fumes, B.H., Andrade, M.A., Franco, M.S. and Lanças, F.M. (2017) On-line approaches for the determination of residues and contaminants in complex samples. Journal of Separation Science 40(1), 183-202.

Gago-Ferrero, P., Schymanski, E.L., Bletsou, A.A., Aalizadeh, R., Hollender, J. and Thomaidis, N.S. (2015) Extended Suspect and Non-Target Strategies to Characterize Emerging Polar Organic Contaminants in Raw Wastewater with LC-HRMS/MS. Environmental science & technology 49(20), 12333-12341.

González-Alonso, S., Merino, L.M., Esteban, S., de Alda, M.L., Barceló, D., Durán, J.J., López-Martínez, J., Aceña, J., Pérez, S. and Mastroianni, N. (2017) Occurrence of pharmaceutical, recreational and psychotropic drug residues in surface water on the northern Antarctic Peninsula region. Environmental Pollution 229, 241-254.

Guan, M., Zhang, X., (2017): Functional genomic assessment of 2,2-bis(bromomethyl)-1,3-propanediol induced cytotoxicity in a single-gene knockout library of E. coli. Chemosphere 185, 582-588.

Guan, M., Fang, W., Ullah, S., Zhang, X., Saquib, Q., Al-Khedhairy, A.A., (2018): Functional genomics assessment of narcotic and specific acting chemical pollutants using E. coli. Environ. Poll. 232: 146-153.

Hashmi, M.A.K., Escher, B.I., Krauss, M., Teodorovic, I. and Brack, W. (2018). Effect-directed analysis (EDA) of Danube River water receiving untreated municipal wastewater in Novi Sad, Serbia. Science of the Total Environment: submitted 1 Nov 2017.

Hilscherova, K., Machala, M., Kannan, K., Blankenship, A. and Giesy, J. (2000) Cell bioassays for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples. Environmental Science and Pollution Research 7, 159-171.

Hug, C., Sievers, M., Ottermanns, R., Hollert, H., Brack, W. and Krauss, M. (2015) Linking mutagenic activity to micropollutant concentrations in wastewater samples by partial least square regression and subsequent identification of variables. Chemosphere 138, 176-182.

Hug, C., Ulrich, N., Schulze, T., Brack, W. and Krauss, M. (2014) Identification of novel micropollutants in wastewater by a combination of suspect and nontarget screening. Environmental Pollution 184(0), 25-32.

Kühnert, A., Vogs, C., Seiwert, B., Aulhorn, S., Altenburger, R., Hollert, H., Küster, E., Busch, W., (2017): Biotransformation in the zebrafish embryo - temporal gene transcription changes of cytochrome P450 enzymes and internal exposure dynamics of the AhR binding xenobiotic benz[a]anthracene. Environ. Poll. 230, 1-11.

Isidori, M., Lavorgna, M., Russo, C., Kundi, M., Žegura, B., Novak, M., Filipič, M., Mišík, M., Knasmueller, S., de Alda, M.L., Barceló, D., Žonja, B., Česen, M., Ščančar, J., Kosjek, T. and Heath, E. (2016) Chemical and toxicological characterisation of anticancer drugs in hospital and municipal wastewaters from Slovenia and Spain. Environmental Pollution 219, 275-287.

Jálová, V., Jarošová, B., Bláha, L., Giesy, J.P., Ocelka, T., Grabic, R., Jurčíková, J., Vrana, B. and Hilscherová, K. (2013) Estrogen-, androgen- and aryl hydrocarbon receptor mediated activities in passive and composite samples from municipal waste and surface waters. Environment International 59(0), 372-383.

König, M., Escher, B.I., Neale, P.A., Krauss, M., Hilscherová, K., Novák, J., Teodorović, I., Schulze, T., Seidensticker, S., Kamal Hashmi, M.A., Ahlheim, J. and Brack, W. (2017) Impact of untreated wastewater on a major European river evaluated with a combination of in vitro bioassays and chemical analysis. Environmental Pollution 220, Part B, 1220-1230.

Krauss, M., Singer, H. and Hollender, J. (2010) LC–high resolution MS in environmental analysis: from target screening to the identification of unknowns. Analytical and bioanalytical chemistry 397(3), 943-951.

Kühnert et al. 2017

Mendoza, A., Rodríguez-Gil, J.L., González-Alonso, S., Mastroianni, N., López de Alda, M., Barceló, D. and Valcárcel, Y. (2014) Drugs of abuse and benzodiazepines in the Madrid Region (Central Spain): Seasonal variation in river waters, occurrence in tap water and potential environmental and human risk. Environment international 70, 76-87.

Moschet, C., Piazzoli, A., Singer, H. and Hollender, J. (2013) Alleviating the Reference Standard Dilemma Using a Systematic Exact Mass Suspect Screening Approach with Liquid Chromatography-High Resolution Mass Spectrometry. Analytical Chemistry 85(21), 10312-10320.

Moschet, C., Wittmer, I., Simovic, J., Junghans, M., Piazzoli, A., Singer, H., Stamm, C., Leu, C. and Hollender, J. (2014) How a Complete Pesticide Screening Changes the Assessment of Surface Water Quality. Environmental Science & Technology 48(10), 5423-5432.

Muz, M., Krauss, M., Kutsarova, S., Schulze, T. and Brack, W. (2017) Mutagenicity in surface waters: synergistic effects of carboline alkaloids and aromatic amines. Environmental science & technology 51(3), 1830-1839.

Myers, A.L., Watson-Leung, T., Jobst, K.J., Shen, L., Besevic, S., Organtini, K., Dorman, F.L., Mabury, S.A. and Reiner, E.J. (2014) Complementary Nontargeted and Targeted Mass Spectrometry Techniques to Determine Bioaccumulation of Halogenated Contaminants in Freshwater Species. Environmental science & technology 48(23), 13844-13854.

Neale, P.A., Ait-Aissa, S., Brack, W., Creusot, N., Denison, M.S., Deutschmann, B.r., Hilscherová, K., Hollert, H., Krauss, M. and Novak, J. (2015) Linking in vitro effects and detected organic micropollutants in surface water using mixture-toxicity modeling. Environmental science & technology 49(24), 14614-14624.

Neale, P.A., Altenburger, R., Ait-Aissa, S., Brion, F., Busch, W., de Aragão Umbuzeiro, G., Denison, M.S., Du Pasquier, D., Hilscherova, K., Hollert, H., Morales, D.A., Novac, J., Schlichting, R., Seiler, T.-B., Serra, H., Shao, Y., Tindall, A.J., Tollefsen, K.E., Williams, T.D. and Escher, B.I. (2017). Development of a bioanalytical test battery for water quality monitoring: Fingerprinting identified micropollutants and their contribution to effects in surface water. Water Research, 123: 734-750.

Neale, P.A., Brack, W., Ait-Aissa, S., Busch, W., Hollender, J., Krauss, M., Maillot-Maréchal, E., Munz, N.A., Schlichting, R., Schulze, T., Vogler, B. and Escher, B. (2018). Solid-phase extraction as sample preparation of water samples for cell-based and other in vitro bioassays. Environ. Sci. Process. Impacts, 20: 493-504.

Neale, P.A., Munz, N.A., Ait-Aissa, S., Altenburger, R., Brion, F., Busch, W., Escher, B.I., Hilscherova, K., Kienle, C., Novak, J., Seiler, T.-B., Shao, Y., Stamm, C. and Hollender, J. (2016). Integrating Chemical Analysis and Bioanalysis to Evaluate the Contribution of Wastewater Effluent on the Micropollutant Burden in Small Streams. Science of the Total Environment: 576: 785-795

Ort et al. 2010

Palma, P., Köck-Schulmeyer, M., Alvarenga, P., Ledo, L., Barbosa, I., De Alda, M.L. and Barceló, D. (2014) Risk assessment of pesticides detected in surface water of the Alqueva reservoir (Guadiana basin, southern of Portugal). Science of the Total Environment 488, 208-219.

Quevauviller, P., Borchers, U., Thompson, C. and Simonart, T. (2008) The water framework directive: ecological and chemical status monitoring, John Wiley & Sons.

Rehberger, K., Werner, I., Hitzfeld, B., Segner, H., Baumann, L., (2017): 20 Years of fish immunotoxicology – what we know and where we are. Crit. Rev. Toxicol. 47(6), 516–542.

Rico, A., Van den Brink, P.J., Leitner, P., Graf W. and Focks A. (2016). Relative influence of chemical and

non-chemical stressors on invertebrate communities: a case study in the Danube River. Science of the Total Environment 571: 1370 – 1382.

Ruttkies, C., Schymanski, E.L., Wolf, S., Hollender, J. and Neumann, S. (2016) MetFrag relaunched: incorporating strategies beyond in silico fragmentation. Journal of Cheminformatics 8(1), 1-16.

Schulze, T., Ahel, M., Ahlheim, J., Ait-Aissa, S., Brion, F., Di Paolo, C., Froment, J., Hidasi, A.O., Hollender, J., Hollert, H., Hu, M., Klolss, A., Koprivica, S., Krauss, M., Muz, M., Oswald, P., Petre, M., Schollee, J.E., Seiler, T.B., Shao, Y., Slobodnik, J., Sonavane, M., Suter, M.J.F., Tollefsen, K.E., Tousova, Z., Walz, K.H. and Brack, W. (2017). Assessment of a novel device for onsite integrative large-volume solid phase extraction of water samples to enable a comprehensive chemical and effect-based analysis. Science of the Total Environment, 581: 350-358.

Schüttler, A., Reiche, K., Altenburger, R., Busch, W., (2017): The transcriptome of the zebrafish embryo after chemical exposure: a meta-analysis. Toxicol. Sci.157, 291-304.

Schwanz, T.G., Llorca, M., Farré, M. and Barceló, D. (2016) Perfluoroalkyl substances assessment in drinking waters from Brazil, France and Spain. Science of the Total Environment 539, 143-152.

Smetanová, S., Riedl, J., Zitzkat, D.P., Altenburger, R., Busch, W., (2015): High-throughput concentration–response analysis for omics datasets. Environ. Toxicol. Chem. 34(9), 2167-2180.

Tousova, Z., Oswald, P., Slobodnik, J., Blaha, L., Muz, M., Hu, M., Brack, W., Krauss, M., Di Paolo, C., Tarcai, Z., Seiler, T.-B., Hollert, H., Koprivica, S., Ahel, M., Schollée, J.E., Hollender, J., Suter, M.J.F., Hidasi, A.O., Schirmer, K., Sonavane, M., Ait-Aissa, S., Creusot, N., Brion, F., Froment, J., Almeida, A.C., Thomas, K., Tollefsen, K.E., Tufi, S., Ouyang, X., Leonards, P., Lamoree, M., Torrens, V.O., Kolkman, A., Schriks, M., Spirhanzlova, P., Tindall, A. and Schulze, T. (2017) European demonstration program on the effect-based and chemical identification and monitoring of organic pollutants in European surface waters. Science of the Total Environment 601–602, 1849-1868.

Wang, P., Xia, P., Yang, J., Wang, Z., Peng, Y., Shi, W., Villeneuve, D.L., Yu, H., Zhang, X., (2018): A Reduced Transcriptome Approach to Assess Environmental Toxicants Using Zebrafish Embryo Test. Environ. Sci. Technol. 52, 821−830.

Zhang, X., Xia, P., Wang, P., Yang, J., Baird, D.J., (2018): Omics Advances in Ecotoxicology. Environ. Sci. Technol. 52, 3842−3851.